EXHIBIT OO

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IN THE UNITED STATES PATENT OFFICE

The Commissioner of Patents,
Washington, D.C. 20231

Sir:

Transmitted herewith for filing is the patent application of inventor: RICHARD AXEL, MICHAEL H WIGLER and SAUL J. SILVERSTEIN

For: PROCESSES FOR INSERTING DNA INTO EUCARYOTIC CELLS AND FOR PRODUCING PROTEINACEOUS MATERIALS

Enclosed are also:

☒ 2 sheets of drawing.

☐ An assignment of the invention to _____

☐ A certified copy of a _____ application.

☐ Associate Power of Attorney.

☐ Preliminary Amendment

Claims as filed

For	Number filed		Number extra	Rate	
Total claims.	125	-10=	115 ×	$2	= 230.00
Independent Claims.	11	- 1=	10 ×	10	= 100.00
Basic Fee					= $65.
Total Filing Fee					= 395.00

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Respectfully,

John P. White

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Declaration and Power of Attorney

We, RICHARD AXEL, MICHAEL H. WIGLER, SAUL J. SILVERSTEIN

declare that we are respectively citizens of The United States of America

residing at 410 Riverside Drive, New York, New York 10025, Cold Spring Harbor Laboratory, P. O. Box 100, Cold Spring Harbor, New York 11724, and 260 Birch Lane, Irvington, New York 10533

that we have read the specification entitled:

PROCESSES FOR INSERTING DNA INTO EUCARYOTIC CELLS AND FOR PRODUCING PROTEINACEOUS MATERIALS

and the claims thereof; that we understand the content of the foregoing specification, and we verily believe we are the original, first, and joint inventors of the invention described and claimed therein; that we do not know and do not believe that this invention was ever known or used in the United States before our invention thereof, or patented or described in any printed publication in any country before our invention thereof, or more than one year prior to this application, or in public use or on sale in the United States more than one year prior to this application; that this invention has not been patented or made the subject of an inventor's certificate in any country foreign to the United States on an application filed by us or our legal representatives or assigns more than twelve months before this application; and that no application for patent or inventor's certificate on this invention has been filed in any country foreign to the United States by us or our representatives or assigns, except as follows:

None

We further declare that we acknowledge our duty to disclose information of which we are aware which is material to the examination of this application.

And we hereby appoint: John P. White (Reg. No. 28678); Jules P. Kirsch (Reg. No. 20293); Thomas F. Moran (Reg. No. 16579); Lester W. Clark (Reg. No. 14954); Christopher C. Dunham (Reg. No. 22031); Ivan S. Kavrukov (Reg. No. 25161) and Charles Guttman (Reg. No. 29161) and each of them, all c/o Cooper, Dunham, Clark, Griffin & Moran of 30 Rockefeller Plaza, New York, New York 10020, (Tel. 212-977-9550), *our attorneys, each with full power of substitution and revocation, to prosecute this application, to make alterations and amendments therein, to receive the patent, and to transact all business in the Patent and Trademark Office connected therewith.*

Lastly we declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

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GENE-CEN 082992

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RESCUE OF pBR FROM TRANSFORMED MOUSE CELLS

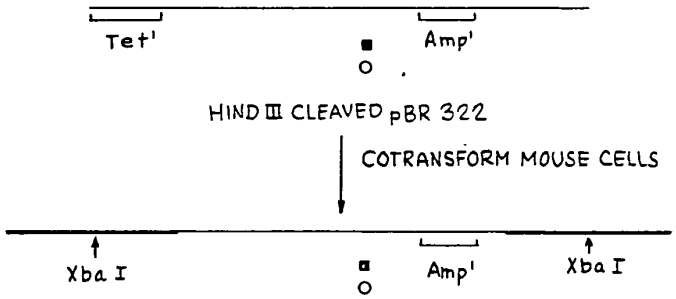

Fig. 2.

SCHEME FOR THE RESCUE OF BACTERIAL PLASMIDS FROM TRANSFORMED CULTURED CELLS USING DOUBLE SELECTION TECHNIQUES.

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COTRANSFORMATION OF EUCARYOTIC CELLS

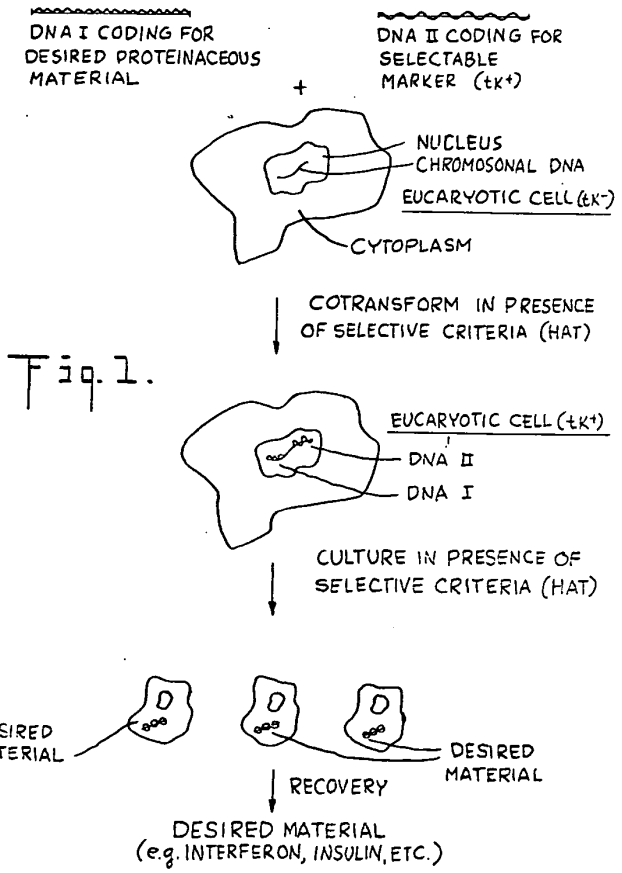

Fig. 1.

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...R INSERTING DNA INTO EUCARYOTIC
...AND FOR PRODUCING PROTEINACEOUS MATERIALS

ABSTRACT OF THE DISCLOSURE

The present invention relates to processes for inserting DNA into eucaryotic cells, particularly DNA which includes a gene or genes coding for desired proteinaceous materials for which no selective criteria exist. The insertion of such DNA molecules is accomplished by cotransforming eucaryotic cells with such DNA together with a second DNA which corresponds to a gene coding for a selectable marker.

This invention also concerns processes for producing proteinaceous materials such as insulin, interferon protein, growth hormone and the like which involve cotransforming eucaryotic cells with DNA which codes for these proteinaceous materials, growing the cotransformed cells for production of the proteinaceous material and recovering the proteinaceous material so produced.

The invention further relates to processes for insertin... into eucaryotic cells a multiplicity of DNA molecules including ~~which includes~~ genes coding for desired proteinaceous materials ~~. The insertion of multiple copies of desired genes is accomplished~~ by cotransformation with the desir... genes and with amplifiable genes for a dominant sele... marker in the presence of successively higher am... an inhibitor. Alternatively, the insertion of m... copies of desired genes is accomplished by transf... using DNA molecules formed by ligating a DNA mole... including the desired gene to a DNA molecule which includes an amplifiable gene coding for a dominant selectable phenotype such as a gen... associated with

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resistance to a drug in the presence of successively higher amounts of an agent such as a drug against which the gene confers resistance so that only those eucaryotic cells into which multiple copies of the amplifiable gene have been inserted survive. ~~Eucaryotic cells into which~~ multiple copies of the amplifiable gene have been inserted additionally include multiple copies of the desired gene and may be used to produce multiple copies of proteinaceous molecules. In this way otherwise rare proteinaceous materials may be obtained in higher concentrations than are obtainable using conventional techniques.

PROCESSES FOR INSERTING DNA INTO EUCARYOTIC CELLS AND FOR PRODUCING PROTEINACEOUS MATERIALS

Field of the Invention

This invention concerns the introduction and expression of genetic informational material, i.e., DNA which includes genes coding for proteinaceous materials and/or genes regulating or otherwise influencing the production thereof, into eucaryotic cells, that is, cells of organisms classified under the Superkingdom Eucaryotes including organisms of the Plant and Animal Kingdoms. Such genetic intervention is commonly referred to as genetic engineering and in certain aspects involves the use of recombinant DNA technology. The invention disclosed is to be distinguished from the introduction of DNA into organisms of the Superkingdom Procaryotes including particularly bacteria. This distinction is based in part upon the basic differences between eucaryotic and procaryotic cells, the former being characterized by true nuclei formed by nuclear envelopes and by meiosis and the latter being characterized by the absence of well-defined nuclei and the absence of meiosis. Moreover, at the genetic level many genes in eucaryotes are split by non-coding sequences which are not continuously colinear, whereas in procaryotes, the genes are continuously colinear.

Background of the Inver

Although advances in the understanding of procaryotic organisms, particularly bacteria, having for the most part proceeded independently of advances in the understanding of eucaryotic organisms, it may be helpful to an appreciation of the present invention to set forth certain developments involving procaryotes.

In 1944, Avery reported the transformation of a procaryotic cell using DNA-mediated transfer of a cellular gene. Avery, O.T., et al., J. Exp. Med. 79: 137-158 (1944). Thereafter, reports of procaryotic transformation occurred in the literature. In 1975, Cohen and others reported results involving first transformation, then cotransformation of the procaryote Escherichia coli. Kretschmer, P.J., et al., J. Bacteriology 124: 225-231 (1975). In the experiments reported therein the authors disclosed the cotransformation of procaryotic cells using plasmid DNA, that is, extrachromosomal DNA which occurs naturally in many strains of Enterobacteriacae. In these experiments it was found that particular cells in a $CaCl_2$-treated bacterial population are preferentially competent for transformation. However, the frequency of transformation and the stability of the transformants obtained was low, possibly because the plasmid is not incorporated into the chromosomal DNA. As a result, cotransformants lost acquired traits after several generations. In addition, these experiments with bacteria required the addition of a gene promoter to the transforming DNA in order to obtain expression.

Meanwhile, experiments with eucaryotic cells proceeded substantially independently of those with procaryotic cells.

In 1962, Szybalska, E.H. and Szybalski, W. PNAS 48: 2026 (1962) reported the transformation of mammalian cells but with such low frequency of transformation that it was not possible to distinguish transformants from cells which had merely undergone spontaneous reversion. Again, as with procaryotic cells, further reports of eucaryotic transformation occurred in the literature, but such results were oftentimes not reproducible by others. In addition, low frequencies of transformation, lack of understanding of the molecular basis for gene expression and the lack of molecular hybridization probes contributed to the lack of progress in this area. As a result, studies on the transformation of eucaryotic cells were essentially restricted to viral genes. Graham, F.L., et al., Cold Spring Harbor Symp. Quant. Biol. 39: 637-650 (1975) and McCutchen, J.H. and Pagano. J.S., Journal National Cancer Institute, 41: 351-357 (1968).

More recently, however, eucaryotic cells, specifically mammalian cells, were transformed with foreign DNA coding for a selectable phenotype. Wigler, M., et al., Cell 11: 223-232 (1977). This work has been extended and has resulted in the present invention wherein it has been discovered inter alia that eucaryotic cells can be cotransformed to yield transformants having foreign DNA integrated into the chromosomal DNA of the eucaryotic cell nucleus. Moreover, it has unexpectedly been discovered that such foreign DNA can be expressed by the cotransformants to generate functional proteins. In addition, by contrast with procaryotic transformants, the foreign DNA is stably expressed through hundreds of generations, a result that may be attributable to integration of the foreign DNA into the chromosomal DNA.

The present invention provides major advances over bacterial systems for future use in the commercial prepar-

ation of proteinaceous materials particularly proteins of eucaryotic origin such as interferon protein, antibodies, insulin, and the like. Such advantages include the ability to use unaltered genes coding for precursors for such proteinaceous materials. After cellular synthesis, the precursor can be further processed or converted within the eucaryotic cell to produce the desired molecules of biological significance. This phenomenon is well known for insulin which is initially produced in the eucaryotic cell as preproinsulin which is then converted to active insulin within the cell by appropriate peptide cleavage. Since procaryotic cells lack the requisite cellular machinery for converting preproinsulin to insulin, the insertion into a procaryotic cell of the eucaryotic gene associated with insulin will result in the production of preproinsulin, not insulin. Although, in the case of insulin, a relatively small and well characterized protein, this difficulty can be overcome by chemical synthesis of the appropriate gene, such an approach is inherently limited by the level of understanding of the amino acid sequence of the desired protein. Thus, for interferon protein, clotting factors, antibodies and uncharacterized enzymes, for which the exact amino acid sequence is not yet known, a procaryotic system will likely not prove satisfactory. By contrast, a eucaryotic system is not associated with such disadvantages since the eucaryotic cell possesses the necessary processing machinery. It is thus one important object of the present invention to provide a process for producing desired proteinaceous materials such as interferon protein, insulin, antibodies and the like which does not require a detailed molecular understanding of amino acid sequence.

In addition to the problem of precursors having additional amino acids which must be removed to produce active protein,

important biological materials may be modified by chemical additions after synthesis and cleavage. Thus, for example, human-produced interferon is a glycoprotein containing sugar molecules in addition to protein. If produced in a bacterial cell, the interferon lacks the sugar molecules which are added when interferon is produced in a human cell. Moreover, proteinaceous materials produced within bacteria may include endotoxins which can cause inflammation if the proteinaceous material is administered to a mammal without significant purification. By contrast, interferon produced in a eucaryotic cell would be free of endotoxins.

It is therefore another important object of this invention to provide a process for producing compounds which include both non-proteinaceous and proteinaceous moieties such as glycoproteins which cannot be produced in bacterial cell.

Summary of the Invention

This invention provides a process for inserting foreign DNA into eucaryotic cells by cotransforming the cells with this foreign DNA and with unlinked DNA which codes for proteinaceous material associated with a selectable phenotype not otherwise expressed by the cell. The cotransformation is carried out in a suitable medium and in the presence of selective conditions permitting survival and/or identification of eucaryotic cells which have acquired the selectable phenotype. The process of this invention is particularly suited for the insertion into eucaryotic cells of DNA which codes for proteinaceous materials which are not associated with a selectable phenotype such as interferon protein, insulin, growth hormones, clotting factors, viral antigens, antibodies and certain enzymes.

By use of the cotransformation process of the present invention is it possible to produce eucaryotic cells which synthesize desired proteinaceous and other materials and which can be grown in culture to produce these materials in quantities not obtainable with conventional technology.

In one embodiment of the invention, the cotransformation process can be used to insert multiple copies of genes coding for desired materials into eucaryotic cells. Alternatively, a multiplicity of foreign DNA molecules corresponding to multiple copies of a desired gene can be inserted into eucaryotic cells by transformation with molecules each of which is formed by linking a foreign DNA molecule to a second DNA molecule corresponding to an amplifiable gene for a dominant selectable

phenotype not otherwise expressed by the cell. The transformation is then carried out in the presence of successively elevated concentrations of an agent permitting survival and/or identification of eucaryotic cells which have acquired multiple copies of the amplifiable gene. This approach is particularly useful when the dominant selectable phenotype is resistance to a drug which is lethal unless multiple copies of the drug resistant gene are present and the agent is the drug.

By inserting multiple copies of genes coding for desired materials into eucaryotic cells according to either of these approaches it is possible to produce eucaryotic cells which yield desired materials in high concentrations and which can be grown in culture to produce such materials in quantities not obtainable with conventional technology.

Brief Description of the Drawings

FIG. 1 is a schematic flow diagram illustrating the cotransformation processs in accordance with the present invention.

FIG. 2 is a schematic flow diagram illustrating a process for recovering foreign DNA I from cotransformed cultured cells using double selection techniques.

Detailed Description of the Invention

Prior to setting forth the invention, it may be helpful to an understanding thereof to set forth definitions of certain terms to be used hereinafter.

Transformation means the process for changing the genotype of a recipient cell mediated by the introduction of purified DNA. Transformation is typically detected by a stable and heritable change in the phenotype of the recipient cell that results from an alteration in either the biochemical or morphological properties of the recipient cell.

Cotransformation means the process for carrying out transformations of a recipient cell with more than one different gene. Cotransformation includes both simultaneous and sequential changes in the genotype of a recipient cell mediated by the introduction of DNA corresponding to either unlinked or linked genes.

Proteinaceous material means any biopolymer formed from amino acids.

Genotype means the genetic constitution of an organism as distinguished from its physical appearance.

Phenotype means the observable properties of an organism as produced by the genotype in conjunction with the environment.

Selectable phenotype is a phenotype which confers upon an organism the ability to exist under conditions which kill off all organisms not possessing the phenotype. Examples include drug resistance or the ability to synthesize some molecule necessary to cell metabolism in a given growth medium. As used herein, selectable phenotypes also include identifiable phenotypes such as the production of materials which pass from or are secreted by the cell and can be detected as new phenotypes either by functional, immunologic or biochemical assays.

Interferon protein means the proteinaceous part of the glycoprotein interferon, that is, the portion remaining after removal of the sugar portion. It includes the protein portion of interferon derived from human leukocyte, fibroblast or lymphoblastoid cells.

Chromosomal DNA means the DNA normally associated with histone in the form of chromosomes residing in the nucleus of a eucaryotic cell.

Transcription means the formation of a RNA chain in accordance with the genetic information contained in the DNA.

Translation means the process whereby the genetic information in an mRNA molecule directs the order of specific amino acids during protein synthesis.

In accordance with the present invention, foreign DNA I can be inserted into any eucaryotic cell by cotransforming the cell with DNA I and with unlinked foreign DNA II which includes a gene coding for a selectable phenotype not expressed by the cell unless acquired by transformation. The cotransformation is carried out in a suitable growth medium and in the presence of selective conditions such that the only cells which survive or are otherwise altered are those which have required the selectable phenotype. See Fig. 1.

Although the experiments discussed hereinafter concern cultured eucaryotic cells of mammalian origin such as human blood cells, mouse fibroblast cells, chinese hamster ovary cells and mouse teratocarcinoma cells, it is clear that the process described is generally applicable to all eucaryotic cells including, for example, cells from birds such as chickens, cells from yeast and fungi, and cells from plants including grains and flowers. Therefore, it is to be understood that the invention encompasses all eucaryotic cells even though the invention may ultimately be most useful in cotransforming mammalian cells.

The present invention is especially useful in connection with the insertion into eucaryotic cells of foreign DNA which includes genes which code for proteinaceous materials not associated with selectable phenotypes. Since such proteinaceous materials are characterized by the fact that they are not associated with a selectable phenotype, cells which contain DNA coding therefore cannot be identified except by destruction of the transformed cell and examination of its contents.

Examples of proteinaceous materials, the genes for which may be inserted into and expressed by eucaryotic cells using the cotransformation process include interferon protein, insulin, growth hormones, clotting factors, viral antigens, enzymes and antibodies.

Although in some cases the DNA I and DNA II may not need to be purified to obtain integration and expression, it is oftentimes preferable that the DNAs be purified prior to use in cotransforming cells. Such purification limits the possibility of spurious results due to the presence of contaminants and increases the probability that cotransformed cells can be identified and stably cultured. Also, although not essential, it is sometimes desirable that DNA I and/or DNA II have been obtained by restriction endonuclease cleavage of chromosomal donor DNAs, such as, for example, restriction endonuclease cleavage of eucaryotic chromosomal DNA. Additionally, it is preferable that DNA I and DNA II be treated with calcium phosphate prior to use in cotransforming eucaryotic cells. The procedure for so treating DNA with calcium phosphate is set forth more fully hereinafter. Finally, it is preferable that the foreign DNA I be present during cotransformation in an amount relative to DNA II coding for a selectable phenotype which constitutes an excess of the former, such as an amount in the range from about 1:1 to about 100,000:1.

In a preferred embodiment of the invention, the foreign DNA I and/or the foreign DNA II are attached to bacterial plasmid or phage DNA prior to use in cotransforming eucaryotic cells. In a particularly promising embodiment, foreign DNA I and/or DNA II are attached to phage DNA and then encapsidated in phage particles prior to cotransformation.

Although any DNA II coding for a selectable phenotype would be useful in the cotransformation process of the present invention, the experimental details set forth particularly concern the use of a gene for thymidine kinase obtained from herpes simplex virus and the use of a gene for adenine phosphoribosyl transferase. In addition, a DNA II which includes a gene coding for a selectable phenotype associated with drug resistance, e.g., a mutant dihydrofolate reductase gene which renders cells resistant to methotrexate greatly extends the applicability of the process.

In accordance with a preferred embodiment, the co-transformation involves DNA I which is physically and chemically unlinked to DNA II, and the DNA I is stably integrated into the chromosomal DNA within the nucleus of the cotransformed eucaryotic cell.

Cotransformation in accordance with this invention may be carried out in any suitable medium limited only in that cotransformed cells be capable of survival and/or identification on the medium. Merely by way of example, a suitable medium for mouse fibroblast cells which have acquired the thymidine kinase gene is HAT described more fully hereinafter. Also, the cotrans-formation is carried out in the presence of selective conditions which permit survival and/or identification of those cells which have acquired the selectable phenotype. Such conditions may include the presence of nutrients, drug or other chemical antagonists, temperature and the like.

Eucaryotic cells cotransformed in accordance with this invention contain foreign DNA I coding for desired materials which can be recovered from the cells using techniques well known in the art. Additionally, the cells can be permitted to transcribe DNA I to form mRNA which in turn is translated to form protein or other desired material which may be recovered, again using well known techniques. Finally, the cells can be grown in culture, harvested and protein or other desired material recovered therefrom.

Although the desired proteinaceous materials identified hereinabove are natural materials, the process can be equally useful in the production of synthetic biopolymers for which synthetic genes are constructed. Thus, the instant invention provides a process for producing novel proteins not yet in existence. Additionally, it provides a process for producing proteins which, although they presently exist, do so in such minute quantities or in such impure form that their isolation and/or identification cannot otherwise be effected. Finally, the invention provides a process for producing partially proteinaceous products such as the glycoproteins and other products, the synthesis of which is genetically directed.

Another aspect of the invention involves processes for inserting multiple copies of genes into eucaryotic cells in order to increase the amount of gene product formed within the cell. One process for inserting a multiplicity of foreign DNA I molecules into a eucaryotic cell comprises cotransforming the cell with multiple DNA I molecules and with multiple, unlinked foreign DNA II molecules corresponding to multiple copies of an amplifiable gene for a dominant selectable

phenotype not otherwise expressed by the cell. This cotransformation process is carried out in a suitable medium and in the presence of an agent permitting survival and/or identification of cells which acquire the dominant selectable phenotype. Preferably, this is done in the presence of successively higher concentrations of such an agent so that only those cells acquiring the highest number of amplifiable dominant genes (DNA II) survive and/or are identified. These cells then also contain multiple copies of DNA I. This approach is particularly appropriate for the insertion of multiple copies of amplifiable genes which confer drug resistance upon the cell, e.g., the mutant dihydrofolate reductase gene which renders cells resistant to methotrexate.

Cotransformed eucaryotic cells which have acquired multiple copies of DNA I may then be used to produce increased amounts of the gene product for which DNA I codes in the same manner as described hereinabove.

Alternatively, multiple copies of foreign genes can be generated in and ultimately expressed by eucaryotic cells by transforming the eucaryotic cells with DNA molecules, each of which has been formed by linking a foreign DNA I to a foreign DNA II which corresponds to an amplifiable gene for a dominant selectable phenotype not normally expressed by the eucaryotic cell. The linkage between DNA I and DNA II is preferably in the form of a chemical bond, particularly a bond formed as a result of enzymatic treatment with a ligase. Transformation with such hybrid DNA molecules so formed is then carried out in a suitable growth medium and in the presence of successively elevated concentrations,

e.g., amounts ranging from 1:1 to 10,000:1 on a molarity basis, of an agent which permits survival and/or identification of those eucaryotic cells which have acquired a sufficiently high number of copies of the amplifiable gene. Using this approach, eucaryotic cells which have acquired multiple copies of the amplifiable gene for a dominant selectable phenotype not otherwise expressed by the cell survive and/or are identifiable in the presence of elevated concentrations of an agent complementary to the amplifiable gene which would otherwise result in death or inability to identify the cells.

Although various amplifiable genes for dominant selectable phenotypes are useful in the practices of this invention, genes associated with drug resistance, e.g., the gene for dihydrofolate reductase which renders cells resistant to methotrexate, are particularly suitable.

By using either of the two approaches just described, multiple copies of proteinaceous or other desired molecules can be produced within eucaryotic cells. Thus, for example, multiple molecules of interferon protein, insulin, growth hormone, clotting factor, viral antigen or antibody or of interferon per se can be produced by eucaryotic cells, particularly mammalian cells, which have been transformed using hybrid DNA or cotransformed using purified DNA which has been treated with calcium phosphate in the manner described hereinafter. Thus, this invention provides a process for producing highly desired, rare and costly proteinaceous and other biological materials in concentrations not obtainble using conventional techniques.

Still another aspect of the present invention involves the preparation of materials normally produced within eucaryotic cells in minute amounts such as glycoproteins including interferon, which are in part protein but additionally include other chemical species such as sugars, ribonucleic acids, histones and the like. Although the method or methods by which cells synthesize complicated cellular materials such as the glycoproteins are poorly understood, it is anticipated that by using the process of the present invention it will be possible to synthesize such materials in commercially useful quantities. Specifically, it is anticipated that after inserting a gene or genes for the protein portion of a cellular material such as a glycoprotein, which includes a non-protein portion, into a eucaryotic cell of the type which normally produces such material, the cell will not only produce the corresponding proteinaceous material but will utilize already existing cellular mechanisms to process the proteinaceous materials, if and to the extent necessary, and will also add the appropriate non-proteinaceous material to form the complete, biologically active material. Thus, for example, the complete biologically active glyprotein, interferon, could be prepared by first synthesizing interferon protein in the manner described and additionally permitting the cell to produce the non-proteinaceous or sugar portion of interferon and to synthesize or assemble true interferon therefrom. The interferon so prepared could then be recovered using conventional techniques.

In accordance with the present invention and as described more fully hereinafter, eucaryotic cells have been stably transformed with precisely defined procaryotic and eucaryotic genes for which no selective criteria exist.

The addition of a purified viral thymidine kinase (tk) gene to mouse cells lacking this enzyme results in the appearance of stable transformants which can be selected by their ability to grow in HAT medium. Since these biochemical transformants might represent a subpopulation of competent cells which are likely to integrate other unlinked genes at frequencies higher than the general population; cotransformation experiments were performed with the viral tk gene and bacteriophage ΦX174, plasmid pBR 322 or cloned chromosomal human or rabbit β-globin gene sequences. Tk transformants were cloned and analyzed for cotransfer of additional DNA sequences by blot hybridization. In this manner, mouse cell lines were identified which contain multiple copies of ΦX, pBR 322, or human and rabbit β-globin sequences. From one to more than 50 cotransformed sequences are integrated into high molecular weight DNA isolated from independent clones. Analysis of subclones demonstrates that the cotransformed DNA is stable through many generations in culture. This cotransformation system allows the introduction and stable integration of virtually any defined gene into cultured eucaryotic cells. Ligation to either viral vectors or selectable biochemical markers is not required.

Cotransformation with dominant-acting markers should in principle permit the introduction of virtually any cloned genetic element into wild-type cultured eucaryotic cells. To this end, a dominant-acting, methotrexate resistant, dihydrofolate reducatse gene from CHO A29 cells was transferred to wild-type cultured mouse cells. By demonstrating the presence of CHO DHFR sequences in transformants, definitive evidence for gene transfer was provided. Exposure of these cells to elevated

levels of methotrexate results in enhanced resistance to this drug, accompanied by amplification of the newly transferred gene. The mutant DHFR gene, therefore, has been used as a eucaryotic vector, by ligating CHO A29 cell DNA to pBR 322 sequences prior to transformation. Amplification of the DHFR sequences results in amplification of the pBR 322 sequences. The use of this gene as a dominant-acting vector in eucaryotic cells will expand the repetoire of potentially transformable cells, no longer restricting these sort of studies to available mutants.

Using the techniques described, the cloned chromosomal rabbit β-globin gene has been introduced into mouse fibroblasts by DNA-mediated gene transfer. The cotransformed mouse fibroblast containing this gene provides a unique opportunity to study the expression and subsequent processing of these sequences in a heterologous host. Solution hybridization experiments in concert with RNA blotting techniques indicate that in at least one transformed cell line rabbit globin sequences are expressed in the cytoplasm as a polyadenylated 9S species. These 9S sequences result from perfect splicing and removal of the two intervening sequences. These results therefor suggest that nonerythroid cells from heterologous species contain the enzymes necessary to correctly process the intervening sequences of a rabbit gene whose expression is usually restricted to erythroid cells. Surprisingly, however, 45 nucleotides present at the 5' terminus of mature rabbit mRNA are absent from the globin mRNA sequence detected in the cytoplasm of the transformants examine. These studies indicate the potential value of cotransformation systems in the analysis of eucaryotic gene expression. The introduction of wild

type genes along with native and in vitro constructed mutant genes into cultured cells provides an assay for the functional significance of sequence organization.

Recombinant DNA technology has facilitated the isolation of several higher eucaryotic genes for which hybridization probes are available. Genes expressed at exceedingly low levels, with mRNA transcripts present at from one to 20 copies per cell, such as those genes coding for essential metabolic functions, cannot be simply isolated by conventinal techniques involving construction of cDNA clones and the ultimate screening of recombinant libraries. An alternative approach for the isolation of such rarely expressed genes has therefore been developed utilizing transformation in concert with a procedure known as plasmid rescue. This schema which is currently underway in the laboratory is outlined below. The aprt gene of the chicken is not cleaved by the enzyme, Hin III or Xba, and transformation of $aprt^-$ mouse cells with cellular DNA digested with these enzymes results in the generation of $aprt^+$ clonies which express the chicken aprt genes. Ligation of Hin III-cleaved chicken DNA with Hin III-cleaved plasmid pBR 322 results in the formation of hybrid DNA molecules in which the aprt gene is now adjacent to plasmid sequences. Transformation of $aprt^-$ cells is now performed with this DNA. Transformants should contain the aprt gene covalently linked to pBR 322 with this entire complex integrated into high molecular weight DNA in the mouse cell. This initial cellular transformation serves to remove the chicken aprt gene from the vast majority of other chick sequences. This transformed cell DNA is now treated with an enzyme, Xba I, which does not cleave either pBR 322 or the aprt gene. The resultant fragments are then circularized with ligase.

One such fragment should contain the aprt gene covalently linked to pBR 322 sequences coding for an origin of replication and the ampicillin resistant marker. Transformation of a bacterium such as E. coli with these circular markers selects for plasmid sequences from eucaryotic DNA which are now linked to chicken aprt sequences. This double selection technique should permit the isolation of genes expressed at low levels in eucaryotic cells for which hybridization probes are not readily obtained.

In order to assist in a better understanding of the present invention, the results of various experiments are now set forth.

EXPERIMENTAL DETAILS

FIRST SERIES OF EXPERIMENTS

The identification and isolation of cells transformed with genes which do not code for selectable markers is problematic since current transformation procedures are highly inefficient. Thus, experiments were undertaken to determine the feasibility of cotransforming cells with two physically unlinked genes. In these experiments it was determined that cotransformed cells could be identified and isolated when one of the genes codes for a selectable marker. Viral thymidine kinase gene was used as a selectable marker to isolate mouse cell lines which contain the tk gene along with either bacteriphage ΦX 174, plasmid pBR 322 or cloned rabbit β-globin gene sequences stably integrated into cellular DNA. The results of these experiments are also set forth in Wigler, M., et al., Cell 16: 777-785 (1979) and Wold, B. et al., Proc. Nat'l. Acad. Sci. 76: 5684-5688 (1979) are as follows:

Experimental Design

The addition of the purified thymidine kinase (tk) gene from herpes simplex virus to mutant mouse cells lacking tk results in the appearance of stable transformants expressing the viral gene which can be selected by their ability to grow in HAT. Maitland, N. J. and McDougall J. K. Cell, 11: 233-241 (1977); Wigler, M. et al., Cell 11: 223-232 (1977). To obtain cotransformants, cultures are exposed to the tk gene in the presence of an excess of a well-defined DNA sequence for which hybridization probes are available. Tk transformants are isolated and scored for the cotransfer of additional DNA sequences by molecular hybridization.

Cotransformation of Mouse Cells with ΦX174 DNA

ΦX174 DNA was initially used in cotransformation experiments with the tk gene as the selectable marker. ΦX replicative form DNA was cleaved with Pst 1, which recognizes a single site in the circular genome. Sanger, F. et al., Nature 265: 687-695 (1977). 500 pg of the purified tk gene were mixed with 1-10 µg of Pst-cleaved ΦX replicative form DNA. This DNA was then added to mouse Ltk$^-$ cells using the transformation conditions described under Methods and Materials hereinafter. After 2 weeks in selective medium (HAT), tk$^+$ transformants were observed at a frequency of one colony per 10 cells per 20 pg of purified gene. Clones were picked and grown to mass culture.

It was then asked whether tk$^+$ transformants also contained ΦX DNA sequences. High molecular weight DNA from the transformants was cleaved with the restriction endonuclease Eco RI, which recognizes no sites in the ΦX genome. The DNA was fractionated by agarose gel electrophoresis and transferred to nitrocellulose filters, and these filters were then annealed with nick-translated ^{32}P-ΦX DNA (blot hybridization). Southern, E. M., J. Mol. Biol. 98: 503-517 (1975); Botchan, M., et al., Cell 9: 269-287 (1976); Pellicer, A., et al. Cell 14: 133-141 (1978). These annealing experiments demonstrate that six of the seven transformants had acquired bacteriophage sequences. Since the ΦX genome is not cut by the enzyme Eco RI, the number of bands observed reflects the minimum number of eucaryotic DNA fragments containing information homologous to ΦX. The clones contain variable amounts of ΦX sequences. Clones ΦX1 and ΦX2 reveal a single annealing fragment which is smaller than the ΦX genome. In these clones, therefore, only a portion of the transforming sequences persist. There

was also observed a tk^+ transformant (clone ΦX3) with no detectable ΦX sequences. Clones ΦX4, 5, 6, and 7 reveal numerous high molecular weight bands which are too closely spaced to count, indicating that these clones contain multiple ΦX-specific fragments. These experiments demonstrate cotransformation of cultured mammalian cells with the viral tk gene and ΦX DNA.

Selection is Necessary to identify ΦX Transformants

It was next asked whether transformants with ΦX DNA was restricted to the population of tk^+ cells or whether a significant proportion of the original culture now contained ΦX sequences. Cultures were exposed to a mixture of the tk gene and ΦX DNA in a molar ratio of 1:2000 or 1:20,000. Half of the cultures were plated under selective conditions, while the other half were plated in neutral media at low density to facilitate cloning. Both selected (tk^+) and unselected (tk^-) colonies were picked, grown into mass culture and scored for the presence of ΦX sequences. In this series of experiments, eight of the nine tk^+ selected colonies contained phage information. As in the previous experiments, the clones contained varying amounts of ΦX DNA. In contrast, none of fifteen clones picked at random from neutral medium contained any ΦX information. Thus, the addition of a selectable marker facilitates the identification of those cells which contain ΦX DNA.

ΦX Sequences are Integrated into Cellular DNA

Cleavage of DNA from ΦX transformants with Eco RI generates a series of fragments which contain ΦX DNA sequences. These fragments may reflect multiple integration events. Alternatively, these fragments could

result from tandem arrays of complete or partial ΦX sequences which are not integrated into cellular DNA. To distinguish between these possibilities, transformed cell DNA was cut with BAM HI or Eco RI, neither of which cleaves the ΦX genome. If the ΦX DNA sequences were not integrated, neither of these enzymes would cleave the ΦX fragments. If the ΦX DNA sequences were not integrated, neither of these enzymes would cleave the ΦX fragments. Identical patterns would be generated from undigested DNA and from DNA cleaved with either of these enzymes. If the sequences are integrated, then BAM HI and Eco RI should recognize different sites in the flanking cellular DNA and generate unique restriction patterns. DNA from clones ΦX4 and ΦX5 was cleaved with BAM III or Eco RI and analyzed by Southern hybridization. In each instance, the annealing pattern with Eco RI fragments differed from that observed with the BAM HI fragments. Furthermore, the profile obtained with undigested DNA reveals annealing only in very high molecular weight regions with no discrete fragments observed. Similar observations were made on clone ΦX1. Thus, the most of the ΦX sequences in these three clones are integrated into cellular DNA.

Intracellular Localization of the ΦX Sequences

The location of ΦX sequences in transformed cells was determined by subcellular fractionation. Nuclear and cytoplasmic fractions was prepared, and the ΦX DNA sequence content of each was assayed by blot hybridization. The data indicate that 95% of the ΦX sequences are located in the nucleus. High and low molecular weight nuclear DNA was prepared by Hirt fractionation. Hirt, B. J., Mol. Biol. 26: 365-369 (1967). Hybridization with DNA from these two fractions indicates that more than 95% of the ΦX information co-purifies with the high molecular

weight DNA fraction. The small amount of hybridization observed in the supernatant fraction reveals a profile identical to that of the high molecular weight DNA, suggesting contamination of this fraction with high molecular weight DNA.

Extent of Sequence Representation of the ΦX Genome

The annealing profiles of DNA from transformed clones digested wtih enzymes that do not cleave the ΦX genome provide evidence that integration of ΦX sequences has occurred and allow us to estimate the number of ΦX sequences integrated. Annealing profiles of DNA from transformed clones digested with enzymes which cleave within the ΦX genome allow us to determine what proportion of the genome is present and how these sequences are arranged following integration. Cleavage of ΦX with the enzyme Hpa I generates three fragments for each integration event: two "internal" fragments of 3.7 and 1.3 kb which together comprise 90% of the ΦX genome, and one "bridge" fragment of 0.5 kb which spans the Pst I cleavage site. In the annealing profile observed when clone ΦX4 is digested with Hpa I, two intense bands are observed at 3.7 and 1.3 kb. A less intense series of bands of higher molecular weight is also observed, some of which probably represent ΦX sequences adjacent to cellular DNA. These results indicate that at least 90% of the ΦX genome is present in these cells. It is worth noting that the internal 1.3 kb Hpa I fragment is bounded by an Hpa I site only 30 bp from the Pst I cleavage site. Comparison of the intensities of the internal bands with known quantities of Hpa I-cleaved ΦX DNA suggests that this clone contains approximately 100 copies of the ΦX genome. The annealing pattern of clone 5 DNA cleaved with

Hpa I is more complex. If internal fragments are present, they are markedly reduced in intensity; instead, multiple bands of varying molecular weight are observed. The 0.5 kb Hpa I fragment which bridges the Pst 1 cleavage site is not observed for either clone ΦX4 or clone ΦX5.

A similar analysis of clone ΦX4 and ΦX5 was performed with the enzyme Hpa II. This enzyme cleaves the ΦX genome five times, thus generating four "internal" fragments of 1.7, 0.5, 0.5 and 0.2 kb, and a 2.6 kb "bridge" fragment which spans the Pst I cleavage site. The annealing patterns for Hpa II-cleaved DNA from ΦX clones 4 and 5 each show an intense 1.7 kb band, consistent with the retention of at least two internal Hpa II sites. The 0.5 kb internal fragments can also be observed, but they are not shown on this gel. Many additional fragments, mostly of high molecular weight, are also present in each clone. These presumably reflect the multiple integration sites of ΦX DNA in the cellular genome. The 2.6 kb fragment bridging the Pst I cleavage site, however, is absent from clone ΦX4. Reduced amounts of annealing fragments which co-migrate with the 2.6 kb Hpa II bridge fragment are observed in clone ΦX5. Similar observations were made in experiments with the enzyme Hae III. The annealing pattern of Hae III-digested DNA from these clones was determined. In accord with previous data, the 0.87 kb Hae III bridge fragment spanning the Pst site is absent or present in reduced amount in transformed cell DNA. Thus, in general, "internal" fragments of ΦX are found in these transformants, while "bridge" fragments which span the Pst I cleavage site are reduced or absent.

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Stability of the Transformed Genotype

Previous observations on the transfer of selectable biochemical markers indicate that the transformed phenotype remains stable for hundreds of generations if cells are maintained under selective pressure. If maintained in neutral medium, the transformed phenotype is lost at frequencies which range from 0.1 to as high as 30% per generation. Wigler, M., et al., Cell 11: 223-232 (1977); Wigler, M. et al., PNAS 76: 5684-5688 (1979). The use of transformation to study the expression of foreign genes depends upon the stability of the transformed genotype. This is an important consideration with genes for which no selective criteria are available. It was assumed that the presence of ϕX DNA in transformants confers no selective advantage on the recipient cell. Therefore, the stability of the ϕX genotype was examined in the descendants of two clones after numerous generations in culture. Clone ϕX4 and ϕX5, both containing multiple-copies of ϕX DNA, were subcloned and six independent subclones from each clone were picked and grown into mass culture. DNA from each of these subclones from each original clone were picked and grown into mass culture. DNA from each of these subclones was then digested with either Eco RI or Hpa I, and the annealing profiles of ϕX-containing fragments were compared with those of the original parental clone. The annealing pattern observed for four of the six ϕX4 subclones is virtually identical to that of the parent. In two subclones, an additional Eco RI fragment appeared which is of identical molecular weight in both. This may have resulted from genotypic heterogeneity in the parental clone prior to subcloning. The patterns obtained for the subclones of ϕX5 are again virtually identical to the parental annealing profile. These data

indicate that ΦX DNA is maintained within the ten subclones examined for numerous generations without significant loss or translocation or information.

Integration of pBR322 into Mouse Cells

The observations in cotransformation have been extended to the EK2-approved bacterial vector, plasmid pBR322. pBR322 linearized with BAM HI was mixed with the purified viral tk gene in a molar ratio of 1000:1. Tk^+ transformants were selected and scored for the presence of pBR322 sequences. Cleavage of BAM HI linearized pBR322 DNA with Bgl I generates two internal fragments of 2.4 and 0.3 kb. The sequence content of the pBR322 transformants was determined by digestion of transformed cell DNA with Bgl I followed by annealing with ^{32}P-labeled plasmid DNA. Four of five clones screened contained the 2.4 kb internal fragment. The 0.3 kb fragment would not be detected on these gels. From the intensity of the 2.4 kb band in comparison with controls, we conclude that multiple copies of this fragment are present in these transformants. Other bands are observed which presumably represent the segments of pBR322 attached to cellular DNA.

Transformation of Mouse Cells with the Rabbit β-Globin Gene

Transformation with purified eucaryotic genes may provide a means for studying the expression of cloned genes in a heterologous host. Cotransformation experiments were therefore performed with the rabbit β major globin gene which was isolated from a cloned library of rabbit chromosomal DNA (Maniatis, T., et al., Cell 15: 687-701 (1978). One β-globin clone designated RβG-1 consists of a

15 kb rabbit DNA fragment carried on the bacteriophage cloning vector Charon 4a. Intact DNA from this clone (RβG-1) was mixed with the viral tk DNA at a molar ratio of 100:1, and tk^+ transformants were isolated and examined for the presence of rabbit globin sequences. Cleavage of RβG-1 with the enzyme Kpn I generates a 4.7 kb fragment which contains the entire rabbit β-globin gene. This fragment was purified by gel electrophoresis and nick-translated to generate a probe for subsequent annealing experiments. The β-globin genes of mouse and rabbit are partially homologous, although we do not observe annealing of the rabbit β-globin probe with Kpn-cleaved mouse DNA under our experimental conditions. In contrast, cleavage of rabbit liver DNA with Kpn I generates the expected 4.7 kb globin band. Cleavage of transformed cell DNA with the enzyme Kpn I generates a 4.7 kb fragment containing globin-specific information in six of the eight tk^+ transformants examined. In two of the clones, additional rabbit globin bands are observed which probably result from the loss of at least one of the Kpn sites during transformation. The number of rabbit globin genes integrated in these transformants is variable. In comparison with controls, some clones contain a single copy of the gene, while others contain multiple copies of this heterologous gene. These results demonstrate that cloned eucaryotic genes can be introduced into cultured mammalian cells by cotransformation.

Transformation Competence Is Not Stably Inherited

Our data suggest the existence of a subpopulation of transformation-competent cells within the total cell population. If competence is a stably inherited trait, then cells selected for transformation should be better recipients in subsequent gene transfer experiments than

their parental cells. Two results indicate that as in procaryotes, competence is not stably heritable. In the first series of experiments, a double mutant, Ltk aprt$^-$ (deficient in both tk and aprt), was transformed to either the tk$^+$ aprt$^-$ or the tk$^-$ aprt$^+$ phenotype using cellular DNA as donor. Wigler, M. et al., Cell 14: 725-731 (1978); Wigler, M. et al., PNAS 76: 5684-5688 (1979). These clones were then transformed to the tk$^+$ aprt$^+$ phenotype. The frequency of the second transformation was not significantly higher than the first. In another series of experiments, clones ΦX4 and ΦX5 were used as recipients for the transfer of a mutant folate reductase gene which renders recipient cells resistant to methotrexate (mtx). The cell line A29 Mtx^{RIII} contains a mutation in the structural gene for dihydrofolate reductase, reducing the affinity of this enzyme for methotrexate. Flintoff, W. F. et al., Somatic Cell Genetic 2: 245-261 (1976). Genomic DNA from this line was used to transform clones ΦX4 and ΦX5 and Ltk$^-$ cells. The frequency of transformation to mtx resistance for the ΦX clones was identical to that observed with the parental Ltk$^-$ cells. It is therefore concluded that competence is not a stably heritable trait and may be a transient property of cells.

Discussion

In these studies, we have stably transformed mammalian cells with precisely defined procaryotic and eucaryotic genes for which no selective criteria exist. Our chosen design derives from studies of transformation in bacteria which indicate that a small but selectable subpopulation of cells is competent in transformation. Thomas, R. Biochim. Biophys. Acta 18: 467-481 (1955); Hotchkiss, R.

PNAS 40: 49-55 (1959); Thomasz, A. and Hotchkiss R. PNAS 51: 480-487 (1964); Spizizen, J. et al., Ann Rev. Microbiol. 20: 371-400 (1966). If this is also true for animal cells, then biochemical transformants will represent a subpopulation of competent cells which are likely to integrate other unlinked genes at frequencies higher than the general population. Thus, to identify transformants containing genes which provide no selectable trait, cultures were cotransformed with a physically unlinked gene which provided a selectable marker. This cotransformation system should allow the introduction and stable integration of virtually any defined gene into cultured cells. Ligation to either viral vectors or selectable biochemical markers is not required.

Cotransformation experiments were performed using the HSV tk gene as the selectable biochemical marker. The addition of this purified tk gene to mouse cells lacking thymidine kinase results in the appearance of stable transformants which can be selected by their ability to grow in HAT. Tk^+ transformants were cloned and analyzed by blot hybridization for cotransfer of additional DNA sequences. In this manner, we have constructed mouse cell lines which contain multiple copies of ΦX, pBR322 and rabbit β-globin gene sequences.

The suggestion that these observations could result from contaminating procaryotic cells in our cultures is highly improbable. At least one of the rabbit β-globin mouse transformants expresses polyadenylated rabbit β-globin RNA sequences as a discrete 9S cytoplasmic species. The elaborate processing events required to generate 9S globin RNA correctly are unlikely to occur in procaryotes.

The ΦX cotransformants were studied in greatest detail. The frequency of cotransformation is high: 14 of 16 tk^+ transformants contain ΦX sequences. The ΦX sequences are integrated into high molecular weight nuclear DNA. The number of integration events varies from one to more than fifty in independent clones. The extent of the bacteriophage genome present within a given transformant is also variable; while some clones have lost up to half the genome, other clones contain over 90% of the ΦX sequences. Analysis of subclones demonstrates that the ΦX genotype is stable through many generations in culture. Similar conclusions are emerging from the characterization of the pBR322 and globin gene cotransformants.

Hybridization analysis of restriction endonuclease-cleaved transformed cell DNA allows one to make some preliminary statements on the nature of the integration intermediate. Only two ΦX clones have been examined in detail. In both clones, the donor DNA was Pst I-linearized ΦX DNA. Attempts were made to distinguish between the integration of a linear or circular intermediate. If either precise circularization or the formation of linear concatamers had occurred at the Pst I cleavage site, and if integration occurred at random points along this DNA, one would expect cleavage maps of transformed cell DNA to mirror the circular ΦX map. The bridge fragment, however, is not observed or is present in reduced amounts in digests of transformed cell DNA with three different restriction endonucleases. The fragments observed are in accord with a model in which ΦX DNA integrates as a linear molecule. Alternatively, it is possible that intramolecular recombination of ΦX DNA occurs, resulting in circularization with deletions at the Pst termini. Lai, C. J. and Nathans, D. Cold Spring Harbor Symp. Quant. Biol. 39: 53-60 (1974).

Random integration of this circular molecule would generate a restriction map similar to that observed for clones ΦX4 and ΦX5. Other more complex models of events occurring before, during or after integration can also be considered. Although variable amounts of DNA may be deleted from termini during transformation, most copies of integrated ΦX sequences in clone ΦX4 retain the Hpa I site, which is only 30 bp from the Pst I cleavage site. Whatever the mode of integration, it appears that cells can be stably transformed with long stretches of donor DNA. Transformants have been observed containing continuous stretches of donor DNA 50 kb long.

There have been attempts to identify cells transformed with ΦX sequences in the absence of selective pressure. Cultures were exposed to ΦX and tk DNA and cells were cloned under nonselective conditions. ΦX sequences were absent from all fifteen clones picked. In contrast, 14 of 16 clones selected for the tk^+ phenotype contained ΦX DNA. The simplest interpretation is that a subpopulation of cells within the culture is competent in the uptake and integration of DNA. In this subpopulation of cells, two physically unlinked genes can be introduced into the same cell with high frequency. At present one can only speculate on the biological basis of competence. Competent cells may be genetic variants within the culture; however, these studies indicate that the competent phenotype is not stably inherited. If one can extrapolate from studies in procaryotes, the phenomenon of competence is likely to be a complex and transient property reflecting the metabolic state of the cell.

Cotransformants contain at least one copy of the tk gene and variable amounts of ΦX DNA. Although transformation was performed with ΦX and tk sequences at a molar ratio of

1000:1, the sequence ratio observed in the transformants never exceeded 100:1. There may be an upper limit to the number of integration events that a cell can tolerate, beyond which lethal mutations occur. Alternatively, it is possible that the efficiency of transformation may depend upon the nature of the transforming fragment. The tk gene may therefore represent a more efficient transforming agent than phage DNA.

In other studies there has been demonstrated the co-transfer of plasmid pBR322 DNA into Ltk$^-$ aprt$^-$ cells using aprt$^+$ cellular DNA as donor and aprt as selectable marker. Furthermore, the use of dominant acting mutant genes which can confer drug resistance will extend the host range for cotransformation to virtually any cultured cell.

The stable transfer of ΦX DNA sequences to mammalian cells serves as a model system for the introduction of defined genes for which no selective criteria exist. The tk cotransformation system has been used to transform cells with the bacterial plasmid pBR322 and the cloned rabbit β-globin gene. Experiments which indicate that several of the pBR transformants contain an uninterrupted sequence which includes the replicative origin and the gene coding for ampicillin resistance (β-lactamase), suggest that DNA from pBR transformants may transfer ampicillin resistance to E. coli. Although preliminary, these studies indicate the potential value of cotrans-formation in the analysis of eucaryotic gene expression.

SECOND SERIES OF EXPERIMENTS

Cotransformed mouse fibroblasts containing the rabbit β-globin gene provide an opportunity to study the expression and subsequent processing of these sequences in a heterologous host. In these experiments, we demonstrate the expression of the transformed rabbit β-globin gene generating a discrete polyadenylated 9S species of globin RNA. This RNA results from correct processing of both intervening sequences, but lacks approximately 48 nucleotides present at the 5' terminus of mature rabbit β-globin mRNA.

Transformation of Mouse Cells with the Rabbit β-Globin Gene

We have performed cotransformation experiments with the chromosomal adult rabbit β-globin gene, using the purified herpes virus tk gene as a biochemical marker. The addition of the tk gene to mutant Ltk^- mouse fibroblasts results in the appearance of stable transformants that can be selected by their ability to grow in hypoxanthine/aminopterin/thymidine (HAT) medium. Cells were cotransformed with a β-globin gene clone designated RβG1, which consists of a 15.5-kbp insert of rabbit DNA carried in the bacteriophage λcloning vector Charon 4A. The purified tk gene was mixed with a 100-fold molar excess of intact recombinant DNA from clone RβG1. This DNA was then exposed to mouse Ltk^- cells under transformation conditions described herein under Methods and Materials. After 2 weeks in selective medium, tk^+ transformants were observed at a frequency of one colony per 10^6 cells per 20 pg of tk gene. Clones were picked and grown into mass culture.

It was then asked if the tk^+ transformants also contain rabbit β-globin sequences. High molecular weight

DNA from eight transformants was cleaved with the restriction endonuclease Knp I. The DNA was fractionated by agarose gel electrophoresis and transferred to nitrocellulose filters, and these filters were then annealed with nick-translated globin [^{32}P] DNA blot hybridization. Southern, E. M., J. Mol. Biol. 98: 503-517 (1975). Cleavage of this recombinant phage with the enzyme Kpn I generates a 4.7-kpb fragment that contains the entire adult β-globin gene, along with 1.4 kbp of 5' flanking information and 2.0 kbp of 3' flanking information. This fragment was purified by gel electrophoresis and nick translated to generate a hybridization probe. Blot hybridization experiments showed that the 4.7-kbp Kpn I fragment containing the globin gene was present in the DNA of six of the eight tk$^+$ transformants. In three of the clones additional rabbit globin bands were observed, which probably resulted from the loss of at least one of the Kpn I sites during transformation. The number of rabbit globin genes integrated in these transformants was variable: some clones contained a single copy of the gene, whereas others contained up to 20 copies of the heterologous gene. It should be noted that the β-globin genes of mouse and rabbit are partially homologous. However, we do not observe hybridization of the rabbit β-globin probe to Kpn-cleaved mouse DNA, presumably because Kpn cleaveage of mouse DNA leaves the β-gene cluster in exceedingly high molecular weight fragments not readily detected in these experiments. These results demonstrate the introduction of the cloned chromosomal rabbit β-globin transfer.

Rabbit β-Globin Sequences are Transcribed in Mouse Transformants

The cotransformation system we have developed may provide a functional assay for cloned eucaryotic genes

if these genes are expressed in the heterologous recipient cell. Six transformed cell clones were therefore analyzed for the presence of rabbit β-globin RNA sequences. In initial experiments, solution hybridization reactions were performed to determine the cellular concentration of rabbit globin transcripts in our transformants. A radioactive cDNA copy of purified rabbit α- and β-globin mRNA was annealed with the vast excess of cellular RNA. Because homology exists between the mouse and rabbit globin sequences, it was necessary to determine experimental conditions such that the rabbit globin cDNAs did not form stable hybrids with mouse globin mRNA but did react completely with homologous rabbit sequences. At 75°C in the presence of 0.4 M NaCl, over 80% hybridization was observed with the rabbit globin mRNA, whereas the heterologous reaction with purified mouse globin mRNA did not exceed 10% hybridization. The $R_0t_{1/2}$ of the homologous hybridization reaction was 6×10^{-4}, a value consistent with a complexity of 1250 nucleotides contributed by the α- plus β-globin sequences in our cDNA probe. Axel, R., et al., Cell 7: 247-254 (1976).

This rabbit globin cDNA was used as a probe in hybridization reactions with total RNA isolated from six transformed cell lines. Total RNA from transformed clone 6 protected 44% of the rabbit cDNA at completion, the value expected if only β-gene transcripts were present. This reaction displayed pseudo-first-order kinetics with $R_0t_{1/2}$ of 2×10^3. A second transformant reacted with an $R_0t_{1/2}$ of 8×10^3. No significant hybridization was observed at R_0ts $\geq 10^4$ with total RNA preparations from the four additional transformants.

We have characterized the RNA from clone 6 in greatest detail. RNA from this transformant was fractionated into nuclear and cytoplasmic populations to determine

the intracellular localization of the rabbit globin RNA. The cytoplasmic RNA was further fractionated by oligo (dT)-cellulose chromatography into poly $(A)^+$ and poly $(A)^-$ RNA. Poly $(A)^+$ cytoplasmic RNA from clone 6 hybridizes with the rabbit cDNA with an $R_0t_{1/2}$ of 25. This value is 1/80th of the $R_0t_{1/2}$ observed with total cellular RNA, consistent with the observation that poly $(A)^+$ cytoplasmic RNA is 1-2% of the total RNA in a mouse cell. Hybridization is not detectable with either nuclear RNA or cytoplasmic poly $(A)^-$ RNA at R_0t values of 1×10^4 and 2×10^4, respectively. The steady-state concentration of rabbit β-globin RNA present in our transformant can be calculated from the $R_0t_{1/2}$ to be about five copies per cell, with greater than 90% localized in the cytoplasm.

Several independent experiments argue that the globin RNA detected derives from transcription of the rabbit DNA sequences present in this transformant: (i) cDNA was prepared from purified 9S mouse globin RNA. This cDNA does not hybridize with poly $(A)^+$ RNA from clone 6 at R_0t values at which the reaction with rabbit globin cDNA is complete (ii) Rabbit globin cDNA does not hybridize with total cellular RNA obtained with tk^+ $globin^-$ transformants at R_0t vlaues exceeding 10^4. (iii) The hybridization observed does not result from duplex formation with rabbit globin DNA possibly contaminating the RNA preparations. Rabbit cDNA was annealed with total cellular RNA from clone 6, the reaction product was treated with S1 nuclease, and the duplex was subjected to equilibrium density centrifugation in cesium sulfate under conditions that separate DNA-RNA hybrids from duplex DNA. The S1-resistant cDNA banded at a density of 1.54 g/ml, as expected for DNA-RNA hybrid structures. These data, along with the observation that globin RNA is poly-

adenylated, demonstrate that the hybridization observed with RNA preparations does not result from contaminating DNA sequences.

Characterization of Rabbit Globin Transcripts in Transformed Cells

In rabbit erythroblast nuclei, the β-globin gene sequences are detected as a 14S precursor RNA that reflects transcription of two intervening sequences that are subsequently removed from this molecule to generate a 9S messenger RNA. It was therefore of interest to determine whether the globin transcripts detected exist at a discrete 9S species, which is likely to reflect appropriate splicing of the rabbit gene transcript by the mouse fibroblast. Cytoplasmic poly (A)-containing RNA from clone 6 was electrophoresed on a methyl-mercury/agarose gel, Bailey, J. & Davidson, N., Anal. Biochem. 70: 75-85 (1976), and transferred to diazotized cellulose paper. Alwine, J. C. et al., Proc. Natl. Acad. Sci. USA 74: 5340-5454 (1977). After transfer, the RNA on the filters was hybridized with DNA from the plasmid pβG1, which contains rabbit β-globin cDNA sequences. Maniatis, T., et al., Cell 8: 163-182 (1976). Using this ^{32}P-labeled probe, a discrete 9S species of RNA was observed in the cytoplasm of the transformant, which comigrated with rabbit globin mRNA isolated from rabbit erythroblasts. Hybridization to 9S RNA species was not observed in parallel lanes containing either purified mouse 9S globin RNA or poly (A)-containing cytoplasmic RNA from a tk$^+$ transformant containing no rabbit globin genes.

In these experiments, it was not possible to detect the presence of a 14S precursor in nuclear RNA populations from the transformants. This is not surprising, because the levels expected in nuclear RNA,

given the observed cytoplasmic concentration, are likely to be below the limits of detection of this techniques. The 5' and 3' boundaries of the rabbit globin sequences expressed in transformed fibroblasts along with the internal processing sites can be defined more accurately by hybridizing this RNA with cloned DNAs, followed by S1 nuclease digestion and subsequent gel analysis of the DNA products. Berk, A. J. & Sharp, P. A., Cell 12: 721-732 (1977). When β-globin mRNA from rabbit erythroid cells was hybridized with cDNA clone p βG1 under appropriate conditions, the entire 576-base pair insert of cDNA was protected from S1 nuclease attack. When the cDNA clone was hybridized with RNA from our transformant, surprisingly, a discrete DNA band was observed at 525 base pairs, but not at 576 base pairs. These results suggest that, in this transformant, rabbit globin RNA molecules are present that have a deletion in a portion of the globin mRNA sequence at the 5' or 3' termini. To distinguish between these possibilities, DNA of the λ clone, R βG1, containing the chromosomal rabbit β- globin sequence hybridized with transformed fibroblast RNA. The hybrid formed was treated with S1 nuclease, and the protected DNA fragments were analyzed by alkaline agarose gel electrophoresis and identified by Southern blotting procedures. Southern, E. M., J. Mol. Biol. 98: 503-517 (1975). Because the rabbit β-globin gene is interrupted by two intervening sequences, the hybridization of mature rabbit mRNA to RβG1 DNA generates three DNA fragments in this sort of analysis: a 146-base pair fragment spanning the 5' terminus to the junction of the small intervening sequence, a 222-base pair internal fragment bridging the small and large intervening sequences, and a 221-base pair fragment spanning the 3' junction of the large intervening sequence to the 3' terminus of the mRNA molecule. When transformant RNA was analyzed in this fashion, a 222-base

pair fragment was observed as well as an aberrant fragment of 100 base pairs but no 146-base pair fragment. Hybridization with a specific 5' probe showed that the internal 222 base pair fragment was present. The sum of the protected lengths equaled the length of the DNA fragment protected by using the cDNA clone. Taken together, these results indicate that although the intervening sequences expressed in transformed mouse fibroblast are removed from the RNA transcripts precisely, the 5' termini of the cytoplasmic transcripts observed do not contain about 48 ± 5 nucleotides present in mature 9S RNA of rabbit erythroblasts.

DISCUSSION

In these studies, mouse cell lines have been constructed that contain the rabbit β-globin gene. The ability of the mouse fibroblast recipient to transcribe and process this heterologous gene has then been analyzed. Solution hybridization experiments in concert with RNA blotting techniques indicate that, in at least one transformed cell line, rabbit globin sequences are expressed in the cytoplasm as a polyadenylylated 9S species. Correct processing of the rabbit β-globin gene has also been observed in tk^+ mouse cell transformants in which the globin and tk plasmids have been ligated prior to transformation. Mantei, N., et al., Nature (London) 281: 40-46 (1970). Similar results have been obtained by using a viral vector to introduce the rabbit globin gene into monkey cells. Hamer, D.H. & Leder, P., Nature (London), 281: 35-39 (1979); Mulligan, R.C., et al., Nature (London) 277: 108-114 (1979). Taken together, these results suggest that nonerythroid cells from heterologous species contain the enzymes necessary to correctly process the intervening sequences of a rabbit gene whose expression usually is restricted to erythroid cells.

The level of expression of rabbit globin sequences in the transformant is low: five copies of globin RNA are present in the cytoplasm of each cell. The results indicate that the two intervening sequences present in the original globin transcript are processed and removed at loci indistinguishable from those observed in rabbit erythroid cells. Surprisingly, 45 nucleotides present at the 5' terminus of mature rabbit mRNA are absent from the β-globin RNA sequence detected in the cytoplasm of the transformant examined. It is possible that incorrect initiation of transcription occurs about the globin gene in this mouse cell line. Alternatively, the globin sequences detected may result from transcription of a long precursor that ultimately must undergo 5' processing to generate the mature 9S species. Incorrect processing at the 5' terminus in the mouse fibroblast could be responsible for the results. At present, it is difficult to distinguish among these alternatives. Because the analysis is restricted to a single transformant, it is not known whether these observations are common to all transformants expressing the globin gene or reflect a rare, but interesting abberation. It should be noted, however, that in similar experiments by Weissman and his colleagues, Mantei, N., et al., Nature (London) 281: 40-46 (1979), at least a portion of the rabbit globin RNA molecules transcribed in transformed mouse fibroblasts retain the correct 5' terminus.

Several alternative explanations can be offered for the expression of globin sequences in transformed fibroblasts. It is possible that constitutive synthesis of globin RNA occurs in cultured fibroblasts, Humphries, S., et al., Cell 7: 267-277 (1976), at levels five to six orders of magnitude below the level observed in erythroblasts. The introduction of 20 additional globin DNA templates may simply increase this constitutive transcription to the levels observed in the transformant. Alternatively, it is possible

that the homologous globin gene is repressed by factors that are partially overcome by a gene dosage effect provided by the introduction of 20 additional globin genes. Finally, normal repression of the globin gene in a fibroblast may depend upon the position of these sequences in the chromosome. At least some of the newly introduced genes are likely to reside at loci distant from the resident mouse globin genes. Some of these ectopic sites may support low level transcription. Present data do not permit one to distinguish among these and other alternatives.

Although the number of rabbit globin genes within a given transformant remains stable for over a hundred generations of culture in hypoxanthine/aminopterin/thymidine, it has not been possible to prove that these sequences are covalently integrated into recipient cell DNA. In previous studies, however, it has been demonstrated that cotransformation of either ΦX174 or plasmid pBR322 results in the stable integration of these sequences into high molecular nuclear DNA. In the present study, the globin gene represents a small internal segment of the high molecular weight concatenated phage DNA used in the transformation. Analysis of integration sites covalently linked to donor DNA is therefore difficult. Preliminary studies using radioactive λ sequences as a probe in DNA blotting experiments indicate that, in some cell lines, a contiguous stretch of recombinant phage DNA with a minimum length of 50 kbp has been introduced.

The presence of 9S globin RNA in the cytoplasm of transformants suggests that this RNA may be translated to give rabbit β-globin polypeptide. Attempts to detect this protein in cell lysates using a purified anti-rabbit β-globin antibody have thus far been unsuccessful. It is possible that the globin RNAs in the transformant are not translated or are translated with very low efficiency due to the ab-

sence of a functional ribosomal binding site. The cytoplasmic globin transcripts in the transformant lack about 48 nucleotides of untranslated 5' sequence, which includes 13 nucleotides known to interact with the 40S ribosomal subunit in nuclease protection studies. Efstratiadis, A., et al., Cell 10: 571-585 (1977); Legon, S., J. Mol. Biol. 106: 37-53 (1976). Even if translation did occur with normal efficiency, it is probable that the protein would exist at levels below the limits of detection of the immunologic assay due to the low level of globin RNA, and the observation that the half-life of β-globin in the absence of heme and globin may be less than 30 min. Mulligan, R.C., et al., Nature (London) 277: 108-114 (1979).

These studies indicate the potential value of cotransformation systems in the analysis of eucaryotic gene expression. The introduction of wild-type genes along with native and in vitro-constructed mutant genes into cultured cells provides an assay for the functional significance of sequence organization. It is obvious from these studies that this analysis will be facilitated by the ability to extend the generality of cotransformation to recipient cell lines, such as murine erythroleukemia cells, that provide a more appropriate environment for the study of heterologous globin gene expression.

THIRD SERIES OF EXPERIMENTS

The cotransformation experiments involving transformation of mouse cells with rabbit β-globin and with plasmid pBR322 and ΦX-174 DNA were continued and extended with the following results.

ΦX DNA was used in cotransformation experiments with the tk gene as the selectable marker. ΦX replicative form DNA was cleaved with Pst I, which recognizes a single site in the circular genome, Sanger, F. et al., Nature 265: 687-695

(1977). Purified tk gene (500 pg) was mixed with 1-10 µg of Pst-cleaved ΦX replicative form DNA. This DNA was then added to mouse Ltk$^-$ cells using the transformation conditions described herein and in Wigler, M., et al., Cell 16:777-785 (1979). After two weeks in selective medium (HAT), tk$^+$ transformants were observed at a frequency of one colony per 10^6 cells per 20 pg of purified gene. Clones were picked and grown into mass culture.

It was then asked whether tk$^+$ transformants contained ΦX DNA sequences. High molecular weight DNA from the transformants was cleaved with the restriction endonuclease Eco RI, which recognizes no sites in the ΦX genome. The DNA was fractionated by agarose gel electrophoresis and transferred to nitrocellulose filters, and these filters were then annealed with nick-translated ^{32}P-ΦX DNA (blot hybridization).

These annealing experiments indicated that 15 of 16 transformants acquired bacteriophage sequences. Since the ΦX genome is not cut with the enzyme Eco RI, the number of bands observed reflects the minimum number of eucaryotic DNA fragments containing information homologous to ΦX. The clones contain variable amounts of ΦX sequences: 4 of the 15 positive clones reveal only a single annealing fragment while others reveal at least fifty ΦX-specific fragments.

It should be noted that none of 15 clones picked at random from neutral medium, following exposure to tk and ΦX DNA, contain ΦX information. Transformation with ΦX therefore is restricted to a subpopulation of tk$^+$ transformants. The addition of a selectable marker therefore facilitates the identification of cotransformants.

Transformation of Mouse Cells with the Rabbit β-Globin Gene

Transformation with purified eucaryotic genes provides a means for studying the expression of cloned genes in a heterologous host. Cotransformation experiments were performed with the rabbit β major globin gene which was isolated from a cloned library of rabbit chromosomal DNA. One β-globin clone, designated R G-1 consists of a 15 kb rabbit DNA fragment carried on the bacteriophage λ cloning vector Charon 4A. Intact DNA from this clone (RβG-1) was mixed with the viral tk DNA at a molar ratio of 100:1, and tk^+ transformants were isolated and examined for the presence of rabbit globin sequences. Cleavage of RβG-1 with the enzyme Kpn I generates a 4.7 kb fragment which contains the entire rabbit β-globin gene. This fragment was purified by gel electrophoresis and nick-translated to generate a probe for subsequent annealing experiments. The β-globin genes of mouse and rabbit are partially homologous, although we do not observe annealing of the rabbit β-globin probe with Kpn-cleaved mouse DNA, presumably because Kpn generates very large globin-specific fragments. In contrast, cleavage of rabbit liver DNA with Kpn I generates the expected 4.7 kb globin band. Cleavage of transformed cell DNA with the enzyme Kpn I generates a 4.7 kb fragment containing globin-specific information in six of the eight tk^+ transformants examined. The number of rabbit globin genes present in these transformants is variable. In comparison with controls, some of the clones contain a single copy of the gene, while others may contain as many as 20 copies of this heterologous gene.

Rabbit β-Globin Sequences are Transcribed in Mouse Transformants

The cotransformation system developed provides a functional assay for cloned eucaryotic genes if these genes are expressed in the heterologous recipient cell. Six transformed cell

clones were analyzed for the presence of rabbit ß-globin RNA sequences. In initial experiments, solution hybridization reactions were performed to determine the cellular concentration of rabbit globin transcripts in transformants.

A radioactive cDNA copy of purified rabbit α and ß-globin mRNA was annealed with a vast excess of total cellular RNA from transformants under experimental conditions such that rabbit globin cDNA does not form a stable hybrid with mouse sequences. Total RNA from transformed clone 6 protects 44% of the rabbit cDNA at completion, the value expected if only ß gene transcripts are present. This reaction displays pseudo-first-order kinetics with an $R_o t_{1/2}$ of 2×10^3. A second transformant (clone 2) reacts with an $R_o t_{1/2}$ of 8×10^3. No significant hybridization was observed with total RNA preparations from four other transformants. Further analysis of clone 6 demonstrates that virtually all of the rabbit ß-globin RNA detected in this transformant is polyadenylated and exists at a steady state concentration of about five copies per cell with greater then 90% of the sequences localized in the cytoplasm.

Globin Sequences Exist as a Discrete 9S Species in Transformed Cells

In rabbit erythroblast nuclei, the ß-globin gene sequences are detected as a 14S precursor RNA which reflects transcription of two intervening sequences which are subsequently spliced from this molecule to generate a 9S messenger RNA. Our solution hybridization experiments only indicate that polyadenylated rabbit globin RNA sequences are present in the mouse transformant. It was therefore of interest to determine whether the globin transcripts we detected exist as a discrete 9S species, which is likely to reflect appropriate splicing of the rabbit gene transcript by the mouse fibroblast. Cytoplasmic poly A-containing RNA from clone 6 was denatured by treatment with 6M urea at 70°C,

and electrophoresed on a 1% acid-urea-agarose gel and transferred to diazotized cellulose paper. Following transfer, the RNA filters were hybridized with DNA from the plasmid RβG-1 containing rabbit β-globin cDNA sequences. Using this ^{32}P-labeled probe, a discrete 9S species of cytoplasmic RNA is seen which co-migrates with rabbit globin mRNA isolated from rabbit erythroblasts. Hybridization to 9S RNA species is not observed in parallel lanes containing either purified mouse 9S globin RNA or polyadenylated cytoplasmic RNA from a tk$^+$ transformant containing no rabbit globin genes.

One is unable in these experiments to detect the presence of a 14S precursor in nuclear RNA populations from the transformant. This is not surprising, since the levels expected in nuclear RNA, given the observed cytoplasmic concentration, are likely to be below the limits of detection of this technique. Nevertheless, the results with cytoplasmic RNA strongly suggest that the mouse fibroblast is capable of processing a transcript of the rabbit β-globin gene to generate a 9S polyadenylated species which is indistinguishable from the β-globin mRNA in rabbit erythroblasts.

Rescue of pBR 322 DNA from Transformed Mouse Cells

Observations on cotransformation were extended to the EK-2 approved bacterial vector, plasmid pBR 322. Using the co-transformation scheme outlined herein, cell lines were constructed containing multiple copies of the pBR 322 genome. Blot hybridization analyses indicate that the pBR 322 sequences integrate into cellular DNA without significant loss of plasmid DNA. pBR 322 DNA linearized with either Hind III or Bam HI, which destroys the tetracycline resistance gene, integrates into mouse DNA with retention of both the plasmid replication origin and the ampicillin resistance (β-lactamase) gene. It was therefore asked whether these plasmid

sequences could be rescued from the mouse genome by a second transformation of bacterial cells.

The experimental approach chosen is outlined in Figure 2. Linearized pBR 322 DNA is introduced into mouse Ltk$^-$ cells via cotransformation using the tk gene as a selectable marker. DNA is isolated from transformants and screened for the presence of pBR 322 sequences. Since the donor plasmid is linearized, interrupting the tetracycline resistant gene, transformed cell DNA contains a linear stretch of plasmid DNA consisting of the replication origin and the β-lactamase gene covalently linked to mouse cellular DNA. This DNA is cleaved with an enzyme such as Xho I, which does not digest the plasmid genome. The resulting fragments are circularized at low DNA concentrations in the presence of ligase. Circular molecules containing plasmid DNA are selected from the vast excess of eucaryotic circles by transformation of E. coli strain χ1776.

This series of experiments has been carried out and a recombinant plasmid isolated from transformed mouse cell DNA which displays the following properties: 1) The rescued plasmid is ampicillin resistant, but tetracycline sensitive consistent with the fact that the donor pBR 322 was linearized by cleavage within the tetracycline resistance gene. 2) The rescued plasmid is 1.9 kb larger than pBR 322 and therefore contains additional DNA. 3) The rescued plasmid anneals to a single band in blot hybridizations to Eco RI-cleaved mouse liver DNA, suggesting that the plasmid contains an insert of single copy mouse DNA. These observations demonstrate that bacterial plasmids stably integrated into the mouse genome via transformation, can be rescued from this unnatural environment, and retain their ability to function in bacterial hosts.

This result immediately suggests modified schemes utilizing plasmid rescue to isolate virtually any cellular gene for which selective growth criteria are available. The aprt gene of the chicken is not cleaved by Hind III or Xho I and transformation of aprt$^-$ mouse cells with cellular DNA digested with these enzymes results in the generation of aprt$^+$ colonies which express the chicken aprt gene. Ligation of Hind III cleaved chicken DNA with Hind III cleaved pBR 322 results in the formation of hybrid DNA molecules, in which the aprt gene is now adjacent to plasmid sequences. Transformation of aprt$^-$ cells is now performed with this DNA. Transformants should contain the aprt gene covalently linked to pBR 322, integrated into the mouse genome. This transformed cell DNA is now treated with an enzyme which does not cleave either pBR 322 or the aprt gene, and the resultant fragments are circularized with ligase. Transformation of E. coli with these circular molecules should select for plasmid sequences from eucaryotic DNA and enormously enrich for chicken aprt sequences. This double selection technique permits the isolation of genes expressed at low levels in eucaryotic cells, for which hybridization probes are not readily obtained.

DISCUSSION

The frequency with which DNA is stably introduced into competent cells is high. Furthermore, the cotransformed sequences appear to be integrated into high molecular weight nuclear DNA. The number of integration events varies from one to greater than fifty in independent transformed clones. At present, precise statements cannot be made concerning the nature of the integration intermediate. Although data with ΦX are in accord with the model in which ΦX DNA integrates as a linear molecule, it is possible that more complex intramolecular recombination events generating circular intermediates may have occurred prior to or during the integration process. Whatever the mode of integration, it

appears that cells can be stably transformed with long stretches of donor DNA. It has been observed that transformants contain contiguous stretches of donor DNA 50 kb long. Furthermore, the frequency of competent cells in culture is also high. At least one percent of the mouse Ltk^- cell recipients can be transformed to the tk^+ phenotype. Although the frequency of transformation in nature is not known, this process could have profound physiologic and evolutionary consequences.

The introduction of cloned eucaryotic genes into animal cells provides an in vivo system to study the functional significance of various features of DNA sequence organization. In these studies, stable mouse cell lines have been constructed which contain up to 20 copies of the rabbit β-globin gene. The ability of the mouse fibroblast recipient to transcribe and process this heterologous gene has been analyzed. Solution hybridization experiments in concert with RNA blotting techniques indicate that in at least one transformed cell line, rabbit globin sequences are expressed in the cytoplasm as a 9S species indistinguishable from the mature messenger RNA of rabbit erythroblasts. These results suggest that the mouse fibroblast contains the enzymes necessary to transcribe and correctly process a rabbit gene whose ~~expresseion~~ expression is normally restricted to erythroid cells. Similar observations have been made by others using a viral vector to introduce the rabbit globin gene into monkey cells.

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These studies indicate the potential value of cotransformation systems in the analysis of eucaryotic gene expression. The introduction of wild type genes along with native and in vitro constructed mutant genes into cultured cells provides an assay for the functional significance of sequence organization. It is obvious from these studies

that this analysis will be facilitated by the ability to extend the generality of cotransformation to recipient cell lines, such as murine erythroleukemia cells, which may provide a more appropriate environment for the study of heterologous globin gene expression.

FOURTH SERIES OF EXPERIMENTS

The ability to transfer purified genes into cultured cells provides the unique opportunity to study the function and physical state of exogenous genes in the transformed host. The development of a system for DNA-mediated transfer of the HSV thymidine kinase (tk) gene to mutant mouse cells, Wigler, M., et al., Cell 11:223-232 (1977), has permitted extension of these studies to unique cellular genes. Wigler, M., et al., Cell 14:725-731 (1979). It has been found that high molecular weight DNA obtained from tk^+ tissues and cultured cells from a variety of eucaryotic organisms can be used to transfer tk activity to mutant mouse cells deficient in this enzyme. The generality of the transformation process has been demonstrated by the successful transfer of the cellular adenine phosphoribosyl transferase (aprt) gene and the hypoxanthine phosphoribosyl transferase (hprt) gene. Wigler, M., et al., Proc. Nat. Acad. Sci. USA 76: 1373-1376 (1979); Willicke, K., et al., Molec. Gen. Genet. 170:179-185 (1979); Graf, L. Y., et al., Somatic Cell Genetics, in press (1979).

More recently, it has been demonstrated that cells transformed with genes coding for selectable biochemical markers also integrate other physically unlinked DNA fragments at high frequency. In this manner, the tk gene has been used as a marker to identify mammalian cells cotransformed with defined procaryotic and eucaryotic genes into cultured mammalian cells. Wigler, M., et al., Cell 16:777-785 (1979).

Detection of gene transfer has in the past relied extensively on the use of appropriate mutant cell lines. In some cases, cells resistant to metabolic inhibitors contain dominant acting mutant genes. Cotransformation with such dominant acting markers should in principle permit the introduction of virtually any cloned genetic element into wild type cultured cells. In this study, cells were transformed with the gene coding for a mutant dihydrofolate reductase (dhfr) gene which renders cells resistant to high concentrations of methotrexate (mtx). Flintoff, W. F., et al., Cell 2:245-262 (1976).

Cultured mammalian cells are exquisitely sensitive to the folate antagonist, methotrexate. Mtx resistant cell lines have been identified which fall into three categories: 1) cells with decreased transport of this drug. Fischer, G. A. Biochem. Pharmacol. 11:1233-1237 (1962); Sirotnak, F. M., et al., Cancer Res. 28:75-80 (1968); 2) cells with structural mutations which lower the affinity of dhfr for methotrexate. Flintoff, W. F., et al., Cell 2:245-262 (1976); and 3) cells which produce inordinately high levels of dhfr. Biedler, J. L., et al., Cancer Res. 32: 153-161 (1972); Chang, S. E., and Littlefield, J. W., Cell 7:391-396 (1976). Where they have been examined, cells producing high levels of dhfr have been found to contain elevated levels of the dhfr gene (gene amplification). Schimke, R. T., et al, Science 202:1051-1055 (1978).

An interesting methotrexate resistant variant cell line (A29) has been identified that synthesizes elevated levels of a mutant dihydrofolate reductase with reduced affinity for methotrexate. Wigler, M., et al., Cell 16:777-785 (1979). Genomic DNA from this cell line has been used as donor in experiments to transfer the mutant dhfr gene to mtx sensitive cells. Exposure of mtx resistant transformed cells to increasing levels of mtx selects for cells which have amplified the transferred gene. In this way, it is possible to trans-

fer and amplify virtually any genetic element in cultured mammalian cells.

Transfer of the Mutant Hamster Dihydrofolate Reductase Gene to Mouse Cells

High molecular weight cellular DNA was prepared from wild-type mtx sensitive CHO cells and from A29 cells, an mtx resistant CHO derivative synthesizing increased levels of a mutant dhfr. Flintoff, W. F., et al., Cell 2:245-262 (1976). The ability of these DNA preparations to transfer either the dhfr gene or the tk gene to tk^- mouse L cells (Ltk^- $aprt^-$) was tested using a modification of the calcium phosphate coprecipitation method. Wigler, M., et al., Proc. Nat. Acad. Sci. USA 76:1373-1376 (1979). DNA from both mutant A29 and wild-type CHO cells was competent in transferring the tk gene to Ltk^- $aprt^-$ cells. Methotrexate resistant colonies were observed only following treatment of cells with DNA from A29. The data obtained suggest that treatment of methotrexate sensitive cells with A29 DNA resulted in the transfer and expression of a mutant dhfr gene, thus rendering these cells insensitive to elevated levels of methotrexate.

In order to test this hypothesis directly, molecular hybridization studies were performed to demonstrate the presence of the hamster dhfr gene in DNA from presumed transformants. A mouse dhfr cDNA clone (pdfr-21), Chang, A.C.Y., et al., Nature 275:617-624 (1978), that shares homology with the structural gene sequences of the hamster dhfr gene was used to detect the presence of this gene in our transformants. Restriction analysis of the dhfr gene from A29, from presumed transformants, and from amplified mouse cells, was performed by blot hybridization. Southern, E. M., J. Mol. Biol. 98:503-517 (1975). DNA was cleaved with restriction endonuclease Hind III, electrophoresed in agarose gels, and transferred to nitrocellulose filters. These filters were

then hybridized with high specific activity, ^{32}P-labeled nick-translated pdhfr-21 and developed by autoradiography. This procedure visualizes restriction fragments of genomic DNA homologous to the dhfr probe. Prominent bands are observed at 15 kb, 3.5 kb and 3 kb for mouse DNA and 17 kb, 7.9 kb, 3.7 kb and 1.4 kb for hamster DNA. The restriction profiles between these two species are sufficiently different to permit one to distinguish the hamster gene in the presence of an endogenous mouse gene. Five L cell transformants resistant to methotrexate were therefore examined by blot hybridization. In each transformed cell line, one observed the expected profile of bands resulting from cleavage of the endogenous mouse dhfr gene and a series of additional bands whose molecular weights are identical to those observed upon cleavage of hamster DNA. The 17.9 kb, 7.9 kb and 1.4 kb bands observed in hamster DNA are diagnostic for the presence of the hamster dhfr gene and are present in all transformants.

In initial experiments, the lowest concentration of methotrexate (0.1 µg per ml) was chosen which would decrease survival of Ltk^- $aprt^-$ cells to less than 10^{-7}. Previous studies, Flintoff, W. F., et al., Cell 2:245-262 (1976), suggested that the presence of a single mutant dhfr gene can render cells resistant to this concentration of methotrexate. Comparison of the intensity of the hamster dhfr gene fragments of transformed cell DNA with those of wild-type hamster DNA suggest that our transformants contain one or at most a few methotrexate resistant hamster genes. By contrast, donor A29 cells, which have been shown to produce elevated levels of the mutant dhfr, Flintoff, W. F., et al., Cell 2:245-262 (1976), appear to contain multiple copies of this gene.

Amplification of the Transferred dhfr Gene

Initial transformants were selected for resistance to relatively low levels of mtx (0.1 µg/ml). For every clone,

however, it was possible to select cells resistant to elevated levels of mtx by exposing mass cultures to successively increasing concentrations of this drug. In this manner, we isolated cultures resistant to up to 40 µg/ml of methotrexate starting from clones that were initially resistant to 0.1 µg/ml. We next asked if increased resistance to methotrexate in these transformants was associated with amplification of a dhfr gene and, if so, whether the endogenous mouse or the newly transferred hamster gene was amplified. DNA from four independent isolates and their resistant derivatives was examined by blot hybridization. In each instance, enhanced resistance to methotrexate was accompanied by an increase in the copy number of the hamster gene. This is most readily seen by comparing the intensities of the 1.5 kb band. In no instance have we detected amplification of the endogenous mouse dhfr gene. Lastly, it is noted that not all lines selected at equivalent methotrexate concentrations appear to have the same dhfr gene copy number.

The dhfr Gene as a Generalized Transformation Vector

Selectable genes can be used as vectors for the introduction of other genetic elements into cultured cells. In previous studies, it has been demonstrated that cells transformed with the tk gene are likely to incorporate other unlinked genes. Wigler, M., et al., Cell 16:777-785 (1979). The generality of this approach was tested for the selectable marker, the mutant dhfr gene. 20 µg of total cellular DNA from A29 was mixed with 1 µg of Hind III-linearized pBR 322 DNA. Recipient cells were exposed to this DNA mixture and, after two weeks, methotrexate resistant colonies were picked. Genomic DNA from transformants was isolated, cleaved with Hind III and analyzed for the presence of pBR322 sequences. Two independent isolates were examined in this way and in both

cases multiple copies of pBR322 sequences were present in these methotrexate transformants.

An alternate approach to generalized transformation involves ligation of a nonselectable DNA sequence to a selectable gene. Since the muant dhfr gene is a dominant acting drug resistance factor, this gene is an ideal vector. Furthermore, it should be possible to amplify any genetic element ligated to this vector by selecting cells resistant to elevated levels of mtx. To explore this possibility, restriction endonucleases that do not destroy the dhfr gene of A29 were identified by transformation assay. One such restriction endonuclease, Sal I, does not destroy the transformation potential of A29 DNA. Sal I-cleaved A29 DNA was therefore ligated to an equal mass of Sal I-linearized pBR322. This ligation product was subsequently used in transformation experiments. Methotrexate resistant colonies were picked and grown into mass culture at 0.1 µg methotrexate/ml. Mass cultures were subsequently exposed to increasing concentrations of methotrexate.

DNAs were obtained from mass cultures resistant to 0.1, 2, 10 and 40 µg/ml methotrexate, and the copy number of pBR322 and dhfr sequences was determined by blot hybridization. Six independent transformed lines were examined in this fashion. Five of these lines exhibited multiple bands homologous to pBR322 sequences. In four of these transformed clones, at least one of the pBR 322-specific bands increased in intensity upon amplification of dhfr. In SS-1, two pBR322-specific bands are observed in DNA from cells resistant to 0.1 µg/ml methotrexate. These bands increase several-fold in intensity in cells resistant to 2 µg/ml. No further increase in intensity is observed, however, in cells selected for resistance to 40 µg/ml. In a second line, SS-6, all pBR 322 bands present at 0.1 µg/ml continue to

increase in intensity as cells are selected first at 2 µg/ml and then at 40 µg/ml methotrexate. Curiously, new pBR322-specific bands appear after selection at higher methotrexate concentrations. It was estimated that there is at least a fifty-fold increase in copy number for pBR322 sequences in this cell line. In a third cell line, HH-1, two pBR322-specific bands increase in intensity upon amplification, others remain constant or decrease in intensity. Thus, the pattern of amplification of pBR322 sequences observed in these cells can be quite varied. Nevertheless, it appears that the mutant dhfr gene can be used as vector for the introduction and amplification of defined DNA sequences into cultured animal cells.

DISCUSSION

The potential usefulness of DNA-mediated transformation in the study of eucaryotic gene expression depends to a large extent on its generality. Cellular genes coding for selectable biochemical functions have previously been introducted into mutant cultured cells, Wigler, M., et al., Cell 14:725-731 (1979); Wigler, M., et al., Proc. Nat. Acad. Sci. USA 76:1373-1376 (1979); Willecke, K., et al., Molec. Gen. Genet. 170:179-185 (1979); Graf, L. H., et al., Somatic Cell Genetics, in press (1979). In the present study, a dominant acting, methotrexate resistant dhfr gene has been transferred to wild-type cultured cells. The use of this gene as a vector in cotransformation systems may now permit the introduction of virtually any genetic element into a host of new cellular environments.

In initial experiments, DNA from A29 cells, a methotrexate resistant CHO derivative synthesizing a mutant dhfr was added to cultures of mouse L cells. Methotrexate resistant colonies appeared at a frequency of one to ten colonies/ 5×10^5 cells/20 µg cellular DNA. No colonies were observed

upon transformation with DNA obtained from wild-type, methotrexate sensitive cells, although this DNA was a competent donor of the thymidine kinase gene. Definitive evidence that we have effected transfer of a mutant hamster dhfr gene was obtained by demonstrating the presence of the hamster gene in mouse transformants. The restriction maps of the mouse and hamster dhfr genes are significantly different and permit one to distinguish these genes in blot hybridization experiments. In all transformants examined, one observes two sets of restriction fragments homologous to a mouse dhfr cDNA clone: a series of bands characteristic of the endogenous mouse gene and a second series characteristic of the donor hamster gene.

The utility of transformation of the dhfr locus is a function of the relative frequencies both of transformation and of spontaneous resistance to mtx. The demonstration that all mtx resistant L cells picked result from transformation rather than amplification of endogenous genes suggests that amplification of dhfr is a rare event in this cell line. Attempts were made to transform other cell lines, including mouse teratoma and rat liver cells and, in these instances, hybridization studies reveal that the acquisition of mtx resistance results from amplification of endogenous dhfr genes. The use of a purified dhfr gene is likely to overcome these difficulties by enormously increasing the frequency of transformation.

The dhfr copy number observed in initial transformants is low. This observation is consistent with previous studies suggesting that a single mutant dhfr gene is capable of rendering cells mtx resistant under selective criteria (0.1 μg/ml mtx). Flintoff, W. F., et al., Cell 2: 245-262 (1976). Exposure of these initial

mtx resistant transformants to stepwise increases in drug concentration results in the selection of cells with enhanced mtx resistance resulting from amplification of the newly transferred mutant hamster dhfr gene. In no transformants has amplification of the endogenous mouse gene been observed in response to selective pressure. It is likely that a single mutant gene affords significantly greater resistance to a given concentration of mtx than a single wild-type gene. If the frequency of the amplification is low, one is merely selecting resistance variants having the minimum number of amplification events. It is also possible that newly transferred genes may be amplified more readily than endogenous genes.

The mutant dhfr gene has been used as a dominant transfer vector to introduce nonselectable genetic elements into cultured cells. One experimental approach exploits the observation made previously, Wigler, M., et al.; Cell 16: 777-785 (1979), that competent cells integrate other physically unlinked genes at high frequency. Cultures exposed to pBR322 DNA, along with the genomic DNA containing the mutant dhfr gene give rise to mtx resistant cell lines containing multiple copies of the bacterial plasmid.

An alternative approach to genetic vectoring involves ligation of pBR322 sequences to the selectable dhfr gene prior to transformations.This procedure also generates transformants containing multiple pBR322 sequences. Amplification of dhfr genes results in amplification of of pBR322 sequences, but the patterns of amplification differ among cell lines. In one instance, all pBR322 sequences amplify with increasing mtx concentrations. In other lines, only a subset of the sequences amplify.

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In yet other lines, sequences appear to have been lost or rearranged. In some lines, amplification proceeds with increasing mtx concentrations up to 40 µg/ml, whereas in others, amplification ceases at 2 µg/ml. At present, the amplification process is not understood nor has the amplification unit been defined. Whatever the mechanisms responsible for these complex events, it is apparent that they can be expolited to control the dosage of virtually any gene introduced into cultured cells.

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FIFTH SERIES OF EXPERIMENTS

Mouse teratocarcinoma (TCC) stem cells provide a unique vector for the introduction of specific, predetermined, genetic changes into mice.Mintz, B. & Illmensee, K., Proc. Natl. Acad. Sci. 72: 3585-3589 (1975); Mintz, B., Brookhaven Symp. Biol. 29: 82-85 (1977). These cells lose their neoplastic properties and undergo normal differentiation when placed in the environment of the early embryo. There they can contribute to formation of all somatic tissues in a mosaic animal comprising both donor- and host-derived cells, and also to the germ line, from which the progeny have genes of the tumor strain in all their cells. Thus, during initial propagation of TCC stem cells in culture, clones with experimentally selected nuclear, Dewey, M. J., et al., Proc.Natl. Acad. Sci., 74 : 5564-5568 (1977), and cytoplasmic, Watanabe, T., et al., Proc. Natl. Acad. Sci., 75: 5113-5117 (1978), gene mutations have been obtained and the cells have proved capable of participating in embryogenesis.

The effective application of this system in probing the control of gene expression during differentiation would be greatly enhanced if, as proposed, Mintz, B., Differentiation 13: 25-27 (1979), precisely defined genes, either in native or modified form, with known associated sequences, could be introduced into developmentally totipotent TCC cells prior to their development in vivo. DNA-mediated gene transfer into cultured mouse cells has now been reported for a variety of viral and cellular genes coding for selectable biochemical functions. The purified viral thymidine kinase (tk; ATP: thymidine 5'-phosphotransferase, EC 2.7.1.21) gene has provided a model system for gene

transfer, Wigler, M. et al., Cell 11: 223-232 (1977), and has been followed by the DNA-mediated transfer of the cellular genes coding for thymidine kinase, Wigler, M., et al., Cell 14: 725-731 (1978), hypoxanthine phosphoribosyltransferase, Willecke, K., et al., Molec. Gen. Genet. 170: 179-185 (1979); Graf, L. H., et al., Somat. Cell Genet., in press (1979), adenine phosphoribosyltransferase, Wigler, M., et al., Proc. Natl. Acad. Sci. USA, 76: 1373-1376 (1979), and dihydrofolate reductase, Wigler, M., et al., Proc. Natl. Acad. Sci, in press (1980); Lewis, W. H., et al., Somat. Cell. Genet., in press (1979). In this report is demonstrated the contransfer of the cloned Herpes simplex (HSV) thymidine kinase gene along with the human β-globin gene into mutant (tk^-) teratocarcinoma stem cells in culture. These transformed cells, when tested by subcutaneous inoculation into mice, retain their developmental capacities in the tumors that are produced, and exhibit the viral-specific tk enzymatic activity for numerous cell generations in vivo.

Transformation of tk^- Teratocarcinoma Cells.

The addition of plasmid DNA containing the HSV thymidine kinase gene to cultures of attached mouse L tk^- cells yields L tk^+ transformants in HAT at a frequency of one colony per 100 pg of DNA per 5 X 10^5 cells. Under identical transformation procedures, tk^- teratocarcinoma cells showed a strikingly lower transformation efficiency. Based on the average of three independent experiments, one surviving colony was obtained per 4 µg of plasmid DNA per 5 X 10^5 cells, a value four to five orders of magnitude below that of the L tk^- cells. This relatively low efficiency was confirmed when the DNA was added to TCC tk^- cells in suspension. Addition of 10 µg of Bam

Hl-restricted ptk-1 DNA to 7 X 10^6 cells resulted in only four transformants in HAT. With identical transformation conditions, L tk^- cells gave 3 X 10^3 tk^+ colonies per 10^7 cells per 1.5 μg of ptk-1 DNA. While high concentrations of gene are thus required to effect transformation in this teratocarcinoma cell line, the availability of cloned DNA nonetheless allows numerous tk^+ transformants to be obtained.

Expression of HSV tk Activity in Transformed Teratocarcinoma Cells.

To ascertain whether the tk^+ phenotypes of the TCC clones were indeed attributable to expression of the viral tk gene, seven colonies were picked from independent culture dishes and grown into mass cultures for testing. The activity of five clones were characterized by serological, and of two by biochemical, techniques. The Herpes-type antigenic identity of tk was verified by assaying the ability of HSV- tk-specific antibody to neutralize enzymatic activity. Over 90% inhibition of tk activity was in fact observed when immune serum was reacted with extracts of each of the five transformed clones chosen (Table I). The low residual activity remaining after neutralization of transformed-cell extracts may represent mitochondrial tk activity, which by itself is unable to afford survival in HAT. Cell extracts from the other two TCC tk^+ clones chosen were tested for tk electrophoretic mobility because of the marked difference between the mouse and HSV tk enzymes. While the TCC tk^- control, as expected, shows no major peak of activity, the transformants have the HSV tk characteristic peak migrating with an R_f of 0.45, as shown for one of the clones.

Table 1. Specific neutralization of Herpes thymidine kinase in transformants

Cell line source of extract	Activity with preimmune serum Units X 10^{-3} per ml	Activity with antiserum Units X 10^{-3} per ml	Activity with antiserum % Residual activity
TCC wt*	2.8	3.0	107.0
TCC tk^{-}†	0.05	0.06	100.0
LHB 2b‡	3.4	0.06	2.0
TCC tk-1§	2.1	0.17	8.0
TCC tk-3	5.5	0.43	8.0
TCC tk-4	6.1	0.15	2.5
TCC tk-5	3.7	0.21	6.0

30,000 X g supernatants of homogenates (S-30) from the indicated cell lines were mixed with preimmune serum or antiserum to purified HSV-1 tk, and tk activity was assayed as described in Materials and Method. Activity is expressed as units per ml of the S-30 fraction.

*TCC wt is a mouse teratocarcinoma feeder-independent cell line (6050P) with tk^{+} (wild-type) phenotype.

†TCC tk^{-} is a derivative of TCC wt that is resistant to BrdUrd and is tk-deficient.

‡LHB 2b is a mouse L tk^{-} cell line transformed to the tk^{+} phenotype with the Herpes thymidine kinase gene.

§TCC tk-1, -3, -4, and -5 are HAT-resistant teratocarcinoma clones derived from TCC tk^{-} after transformation with the Herpes thymidine kinase gene.

The Physical State of the tk Gene in Transformed Teratocarcinoma Cells

The number of viral tk gene fragments and the location of these fragments in independent transformants were examined utilizing the blot hybridization technique of Southern, Southern, E. M., J. Mol. Biol., 98: 503-517 (1975). The donor DNA was the recombinant plasmid, ptk-1, digested to completion with Bam Hl. This plasmid contains a 3.4 kb fragment with the viral tk gene inserted at the single Bam Hl site within the tetracycline resistance gene of pBR322. Transformation with Bam-cleaved tk DNA results in integration with loss of the Bam sites at the termini of the 3.4 kb fragment. High molecular weight DNA from transformants was cleaved with Bam Hl, fractionated by agarose gel electrophoresis, and transferred to nitrocellulose filters; the filters were then annealed with nick-translated ^{32}P-tk DNA. In each cell clone, a single annealing fragment was seen; therefore, each clone contains at least one viral tk gene. As expected, each clone reveals a band of molecular weight greater than 3.4 kb. The molecular weights of the annealing fragments differ among the transformed clones, a result suggesting that integration has occurred at different sites within the DNA of the respective transformants.

Stability of the Transformed Phenotype in Culture

To test the capacity of the TCC transformants to retain expression of the donor tk gene in culture in the absence of selective pressure, individual clones grown into mass culture in HAT selective medium were subcultured for various periods in the absence of the selective agent. The fraction of cells that retained the tk$^+$ phenotype was determined by measuring cloning efficiencies in selective and nonselective media. Wide differences among clones became apparent (Table II). Some cell lines,

Table II. In vitro stability of the transformed phenotype in teratocarcinoma cells.

Clonal cell line	Experiment	Generations in nonselective medium*	Relative cloning efficiency in selective medium†	Rate of loss of tk^+ phenotype per generation‡
TCC tk-1	1	28	0.45	
	2	150	0.50	<0.001
TCC tk-2	1	28	0.23	
	2	150	0.02	0.017
TCC tk-3	1	28	0.47	
	2	150	0.27	0.002
TCC tk-4	1	28	0.26	
	2	150	0.16	0.003
TCC tk-5	1	28	0.14	
	2	150	0.01	0.021

*Clones were picked and grown in HAT selective medium for 40 cell generations. Cells were then grown in nonselective medium for 28 or 150 generations prior to determining their cloning efficiencies under selective and nonselective conditions.

†One hundred cells were plated in triplicate into HAT selective and nonselective media. The relative cloning efficiency in selective medium is defined as the ratio of the cloning efficiency under selective conditions to the cloning efficiency under nonselective conditions (50-70%).

‡In these calculations it is assumed that for any given cell line the rate of loss of the tk phenotype is constant in each cell generation. The rate of loss per generation may then be calculated from the formula $F_M (1-X)^{N-M} + F_N$, in which F_M is the relative cloning efficiency in selective medium after M generations in non-selective medium; F_N is similarly defined for N generations; and X is the rate of loss per cell generation.

such as TCC tk-1, were relatively stable and lost the tk^+ phenotype at frequencies less than 0.1% per generation in nonselective medium. Other, less stable, lines (TCC tk-2 and TCC tk-5) lost tk^+ expression at 2% per generation in the absence of selection.

Maintenance and Expression of the HSV tk Gene in Vivo During Tissue Differentiation in Tumors

The more critical question of retention of the foreign gene and of its expression during TCC cell differentiation in vivo in the absence of selection was examined in solid tumors. Tumors were formed by inoculating syngeneic hosts (usually two hosts per clone) subcutaneously with 10^7 cells from each of the same five transformed clones. DNA from these tumors was analyzed by blot hybridization. Neutralization assays and electrophoretic mobility tests of the tk enzyme were also carried out to identify expression of the viral gene. In addition, samples of the same tumors were fixed and examined histologically for evidence of differentiation.

The restriction fragment profiles of the viral tk gene demonstrated that the gene was retained in all nine tumors analyzed. When each tumor (grown without HAT selection) was compared with its cell line of origin (cultured under HAT selective pressure), the number and location of the annealing fragments in seven of the tumors was identical to that of the corresponding cell line. Thus, the introduced tk gene was, in most cases, maintained for many cell generations spanning at least three weeks in vivo without significant loss or translocation. In two instances, however, a gene rearrangement had occurred, resulting from the loss of the original tk-containing fragment and the appearance of a new fragment of different molecular weight. It is of interest that

these two tumors were produced from the two TCC clones that lost the tk^+ phenotype in vitro at highest frequencies (Table II).

The results of neutralization tests with HSV-tk-specific antiserum demonstrated that at least three of the nine tumors (including one from the TCC tk-1 clone) had viral-type tk activity. (The presence of host cells in the tumors probably contributed substantial amounts of non-neutralized mouse tk in the remaining cases.) Another sample of the tumor derived from the TCC tk-1 line was also analyzed electrophoretically for HSV tk activity; a predominant peak migrating with an R_f of 0.45, characteristic of the viral enzyme, was observed.

Histological speciments from each of the tumors were prepared and examined. In addition to the TCC stem cells, tumors contained an array of differentiated tissues similar to those in tumors from the untransformed TCC wt and TCC tk^- cell lines of origin. Included were muscle, neural formations, adipose tissue, some bone, squamous keratinizing epithelium, and other epithelia, ducts, and tubules.

Cotransformation of Teratocarcinoma Cells with the Human β-Globin Gene

Biochemical transformants of mouse L may constitute a competent subpopulation in which an unselectable gene can be introduced, along with an unlinked selectable gene, at frequencies higher than in the general population, Wigler, M., et al., Cell 16: 777-785 (1979). Cotransformation experiments have therefore been carried out in which the Herpes viral tk gene was used as a

selectable marker to introduce the human β-globin gene into tk$^-$ TCC cells. A cloned Hind III restriction endonuclease fragment of human chromosomal DNA containing the β-globin gene (plasmid phβ-8) was cleaved with the enzyme Hind III and mixed with Hind III-linearized ptk-1. After TCC tk$^-$ cells were exposed to these genes, they were grown for two weeks in HAT selection medium and tk$^+$ transformants were cloned and analyzed by blot hybridization for presence of human β-globin sequences. A 4.3 kb Bgl II restriction fragment containing the intact human β-globin gene is entirely contained within the donor pH -8 plasmid. High molecular weight DNA from the transformants was therefore cleaved with the Bgl II enzyme and analyzed in blot hybridization using the ^{32}P-labeled 4.3 kb Bgl II fragment as an annealing probe.

In two of the ten TCC transformants examined, human β-globin sequences were detected. One of the transformants contains one to three copies of the 4.3 kb Bgl II fragment; in this cell line, therefore, the globin gene is evidently intact. The other TCC isolate containing the human β-globin gene displays an aberrant high molecular weight annealing fragment, a result suggesting that cleavage and integration have occurred within the Bgl II fragment. These data demonstrate that those TCC cells that are competent for uptake and expression of the tk gene also integrate another unlinked and unselectable gene at high frequency.

DISCUSSION

The experimental introduction of foreign DNA into early mammalian embryos, and its persistence and augmentation during development, were first reported some six years

ago, Jaenisch, R. & Mintz, B., Proc. Natl. Acad. Sci. 71: 1250-1254 (1974). Purified (nonrecombinant) SV 40 viral DNA was microinjected into mouse blastocysts; they gave rise to healthy adults whose tissue DNA contained SV 40 gene sequences. Newer technologies such as described herein should allow a wide range of specific genes to be incorporated into the genome of the embryo for in vivo analyses of control of gene expression during differentiation. With the advent of recombinant DNA, quantities of particular genes in native or specifically modified form can be obtained. In the biological sphere, the malignant stem cells of mouse teratocarcinomas have contributed a novel avenue of intervention. These cells can be grown in culture, selected for specific mutations, and microinjected into blastocysts, where they lose their neoplastic properties and participate in development, Dewey, M., J. et al., Proc. Natl. Acad, Sci. USA, 74: 5564-5568 (1977); Watanabe, T., et al., Proc. Natl. Acad. Sci., 75: 5113-5117 (1978). The cultured TCC cells have therefore been viewed as vehicles for transmitting predetermined genetic changes to mice, Mintz, B., Brookhaven Symp., Bio., 29: 82-85, (1977); Mintz, B., Differentiation 13: 25-27 (1979). Such changes obviously might include genes acquired by uptake of DNA.

DNA-mediated gene transfer into cells of fibroblast lines has been accomplished in culture, Wigler, M., et al., Cell 11: 223-232 (1977); Wigler, M., et al., Cell 14: 725-731 (1978); Willecke, K., et al., Molec. Gen. Genet. 170: 179-185 (1979), Graf, L. H., et al., Somat. Cell Genet., in press (1979); Wigler, M., et al., Proc. Natl. Acad. Sci. USA, 76: 1373-1376 (1979); Wigler, M., et al. Proc. Natl. Acad. Sci., in press (1980); Lewis, W. H. et al., Somat. Cell Genet., in press (1979), and furnished the basis for similar attempts here with tera-

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tocarcinoma lines. The TCC-cell route for gene transfer into embryos, as compared with embryo injection of DNA, offers the advantage that transformants, i.e., cell clones in which the specific gene has been retained, can be identified and isolated by selection or screening. In the case of unselectable genes, cotransfer with a selectable one has been found to occur with relatively high frequency, Wigler, M., et al., Cell 16: 777-785 (1979).

In the present study, tk^- teratocarcinoma cells have been treated with the cloned thymidine kinase gene of Herpes simplex and a number of HAT-resistant tk+ clones have been obtained with a frequency of about one transformant per µg of DNA. The reason for the markedly lower frequency of TCC transformants than of L-cell transformants, Wigler, M., et al., Cell 14: 725-731 (1978), is obscure since the basis for transformation competence in eucaryotic cells remains unknown. The donor origin of the tk^+ phenotype in the TCC transformants was demonstrated by the HSV-type electrophoretic mobility of their tk enzyme, and also by neutralization of the tk activity by specific antiserum raised against HSV-1 tk (Table I). Furthermore, blot hybridization tests indicated that at least one intact copy of the viral tk gene was present and integrated into other DNA in the transformed cells. These data support the conclusion that the tk activity in the transformed clones is indeed attributable to presence and expression of the viral gene

A requirement for experiments involving the introduction of genes is that they remain stable in vivo, even in the absence of selective pressure, during many cell generations. Stability of the tk^+ transformed phenotype was in fact not only in culture (Table II), but also in tumors arising

after subcutaneous inoculation of the stem cells into mice. These tumors exhibited various types of tissue differentiation, similar to the range observed in the untransformed parent TCC line. Hybridization experiments comparing each tumor with its transformed cell line of origin indicated that the donor tk gene was maintained without significant loss or rearrangement in seven of nine tumors examined.

Many genes of interest in a developmental context are not selectable. An example is the globin gene. As in related experiments with L-cells, Wigler, M., et al., Cell 16: 777-785 (1979), a fragment of human genomic DNA containing an intact β-globin gene was administered to TCC tk^- cells along with the unlinked HSV tk gene. This proved to be an effective method to obtain TCC tk^+ clones in which, from hybridization evidence, the human β-globin gene was present.

The experiments described herein therefore demonstrate that cultured TCC stem cells can accept exogenous genes and that such genes can be stably retained as well as expressed during in vivo differentiation in tumors. On this basis, experiments with a euploid TCC cell line can proceed, for the purpose of creating in vivo markers appropriate for analyses of gene regulation during embryogenesis.

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MATERIALS AND METHODS

Cell Cultures

Ltk aprt, a derivative of Ltk clone D, Kit, S. et al., Esp. Cell Res. 31:291-312 (1963), was maintained in Dulbecco's modified Eagle's medium (DME) containing 10% calf serum (Flow Laboratories, Rockville, Maryland) and 50 µg/ml of diaminopurine (DAP). Prior to transformation, cells were washed and grown for three generations in the absence of DAP. A Chinese hamster cell line containing an altered dihydrofolate reductase (rendering it resistant to methoxtrexate) A29 Mtx^{RIII}, Flintoff, W. F., et al., Somatic Cell Genetics 2:245-261 (1976), was propagated in DME supplemented with 3x non-essential amino acids, 10% calf serum and 1 µg/ml amethopterin. For the amplification experiments, the medium was additionally supplemented with 20 µg/ml of methotrexate.

Murine Ltk^- $aprt^-$ cells are adenine phosphoribosyltransferase-negative derivatives of Ltk^- clone D cells. Cells were maintained in growth medium and prepared for transformation as described, Wigler, M., et al., PNAS 76:1373-1376 (1979).

HEp-2(human), HeLa(human), CHO (Chinese hamster ovary), and Ltk^- cells were grown in growth medium. LH2b, a derivative of Ltk^- transformed with herpes simples virus tk DNA, was maintained in growth medium containing hypoxanthine at 15 µg/ml, aminopterin at 0.2 µg/ml, and thymidine at 5.0 µg/ml (HAT), Wigler, M., et al., Cell 1:223-232 (1977). All culture dishes were Nunclon (Vanguard International, Neptune, N. J.) plastic.

The feeder-independent mouse teratocarcinoma cell culture line 6050P, Watanabe, T., et al., PNAS 75:5113-5117 (1978),

obtained from a tumor of the OTT 6050 transplant line, was used as the wild-type, or tk^+, parent and is here designated TCC wt. This line is of the X/O sex chromosome type and has a modal number of 39 chromosomes with characteristics described in Watanabe, T., et al., (1978). The cells were grown in Dulbecco's modified Eagle's medium with 10% fetal calf serum. After 3 hr of exposure to 3 µg/ml of the mutagen N-methyl-N'-nitro-N-nitrosoguanidine, the cells were allowed to recover for two days and were then transferred to medium with 80 µg/ml of BrdUrd. A series of resistant clones were isolated; one supplied the clonal line (TCC tk^-) used in the present transformation experiments. This line had a reversion frequency to wild-type of less then 10^{-8}. The cells were maintained in medium with 30 µg/ml of BrdUrd and, prior to transformation, were washed and grown for three generations in the absence of the drug. Transformation efficiency was compared with that of a tk-deficient line, Kit, S., et al., Exp. Cell. Res. 31:297-312 (1963) of mouse L-cells (L tk^-).

Extraction and Restriction Endonuclease Cleavage of Genomic DNA

High molecular weight DNA was obtained from cultured cells (CHO, LH2b, and HeLa) or from frozen rabbit livers as previously described. Wigler, M., et al., Cell 14:725-731 (1978). High molecular weight salmon sperm DNA was obtained from Worthington. Restriction endonuclease cleavage (Bam I, HindIII, Kpn I, and Xba I) was performed in a buffer containing 50 mM NaCl, 10 mM Tris·HCL, 5 mM $MgCl_2$, 7 mM mercaptoethanol, and bovine serum albumin at 100 µg/ml (pH 7.9). The enzyme-to-DNA ratio was at least two units/µg of DNA, and reaction mixtures were incubated at 37°C for at least 2 hrs (one unit is the amount of enzyme that digests 1 µg of DNA in 1 hr). To monitor the completeness of digestion, 1 µl of nick-translated adenovirus-2 [^{32}P]DNA was incubated

with 5 µl of reaction volume for at least 2 hr, cleavage products were separated by electrophoresis in 1% agarose gels, and digestion was monitored by exposing the dried gel to Cronex 2DC x-ray film.

Intact herpes simplex virus (HSV) DNA was isolated from CV-1-infected cells as previously described. Pellicer, A., et al., Cell 14:133-141 (1978). DNA was digested to completion with Kpn I (New England Biolabs) in a buffer containing 6 mM Tris (pH 7.9), 6mM $MgCl_2$, 6 mM 2-mercaptoethanol, 6 mM NaCl and 200 µg/ml bovine serum albumin. The restricted DNA was fractionated by electrophoresis through 0.5% agarose gels (17 x 20 x 0.5 cm) for 24 hr at 70 V, and the 5.1 kb tk-containing fragment was extracted from the gel as described by Maxam, A. M. and Gilbert, W. PNAS 74:560-564 (1977) and Wigler, M., et al., Cell 14:725-731 (1978)

φX174 am3 RFI DNA was purchased from Bethesda Research Laboratories. Plasmid pBR322 DNA was grown in E. coli HB 101 and purified according to the method of Clewell, D. B., J. Bacteriol. 110:667-676 (1972). The cloned rabbit β major globin gene in the λ Charon 4A derivative (RβG-1) was identified and isolated as previously described. Maniatis, T., et al., Cell 15:687-701(1978).

In the amplification experiments, the size of the high molecular weight DNA was determined by electrophoresis in 0.3% agarose gels using herpes simplex virus DNA and its Xba I fragments as markers. Only DNA whose average size was larger than 75 kb was found to possess transforming activity in the amplification experiments. In these experiments, plasmid DNAs were isolated from chloramphenicol amplified cultures by isopycnic centrifugation in CsCl gradients containing 300 µg/ml ethidium bromide.

Transformation and Selection

The transformation protocol was as described in Graham, F. L. and Van der Eb, A. J., Virology, 52:456-457 (1973) with the following modifications. One day prior to transformation, cells were seeded at 0.7 X 10^6 cells per dish. The medium was changed 4 hr prior to transformation. Sterile, ethanol-precipitated high molecular weight or restriction endonuclease-cleaved eucaryotic DNA dissolved in 1 mM Tris (pH 7.9)/0.1 mM EDTA was used to prepare DNA/$CaCl_2$ which contains DNA at 40 µg/ml and 250 mM $CaCl_2$ (Mallinkrodt). Twice-concentrated Hepes-buffered saline (2X HBS) was prepared; it contains 280 mM NaCl, 50 mM Hepes, and 1.5 mM sodium phosphate, pH adjusted to 7.10 ± 0.05. DNA/$CaCl_2$ solution was added dropwise to an equal volume of sterile 2X HBS. A 1-ml sterile plastic pipette with a cotton plug was inserted into the mixing tube containing 2X HBS, and bubbles were introduced by blowing while the DNA was being added. The calcium phosphate/DNA precipitate was allowed to form without agitation for 30-45 min at room temperature. The precipitate was then mixed by gentle pipetting with a plastic pipette, and 1 ml of precipitate was added per plate, directly to the 10 ml of growth medium that covered the recipient cells. After 4-hr incubation at 37°C, the medium was replaced and the cells were allowed to incubate for an additional 20 hr. At that time, selective pressure was applied. For tk^+ selection, medium was changed to growth medium containing HAT. For $aprt^+$ selection, cells were trypsinized and replated at lower density (about 0.5 X 10^6 cells per 10-cm dish) in medium containing 0.05 mM azaserine and 0.1 mM adenine. For both tk^+ and $aprt^+$ selection, selective media were changed the next day, 2 days after that, and subsequently every 3 days for 2-3 weeks while transformant clones arose. Colonies were picked by using cloning cylinders and the remainder of the colonies were scored after formaldehyde fixation and staining with Giemsa. For

characterization, clones were grown into mass culture under continued selective pressure. A record was kept of the apparent number of cell doublings for each clone isolated.

Methotrexate-resistant transformants of Ltk$^-$ aprt$^-$ cells were obtained following transformation with 20 µg of high molecular weight DNA from A29 Mtx^{RIII} cells and selection in DME containing 10% calf serum and 0.2 µg/ml amethopterin.

For tk$^+$ selection, cells were grown in HAT medium; for resistance to methotrexate, cells were selected in medium supplemented with 0.1 µg/ml of methotrexate. Colonies were cloned from individual dishes to assure that each transformant arose from an independent event. Ligates between A29 DNA and linearized pBR322 DNA were prepared by incubating a 1:1 ration(w/w) of Sal I-cleaved DNAs with T_4 ligase (Bethesda Research Laboratories) under the conditions recommended by the supplier. A calcium phosphate precipitate was prepared using 2 µg ligate and 18 µg carrier/ml, and added to recipient cells (the amount of ligate was limited because of the observation that plasmid inhibits transformation). The DNA was allowed to remain in contact with the cells for 4-12 hr and the medium was then aspirated and replaced with fresh DME. Selective pressure was applied 24 hr following exposure to DNA. After 2-3 weeks, colonies were isolated using cloning cylinders.

In the mouse teratocarcinoma cell experiments, transformation was performed as described previously except that the TCC tk$^-$ cells were seeded at 3 X 10^5 cells/plate one day prior to transformation. To each plate of attached cells was added a calcium phosphate/DNA precipitate prepared with 4 µg of the recombinant plasmid, Ptk-1, digested with Bam Hl, in the presence of 20 µg of high molecular weight DNA obtained from L tk$^-$ aprt$^-$ cells.

In addition, some cells were treated in suspension, Willecke, K. et al., Molec. Gen. Genet. 170:179-185 (1979). 7×10^6 freshly trypsinized TCC tk^- cells were mixed with a calcium phosphate/DNA precipitate prepared with 10 µg of DNA from the Bam Hl-cleaved plasmid Ptk-1 and 150 µg of high molecular weight DNA from salmon sperm. Following centrifugation, resuspension, and shaking, as described in Willecke, K. et al. (1979), the cells were again plated in growth medium. After three days, the medium was replaced with HAT medium and colonies of transformants were isolated after two weeks.

Cotransformation experiments were performed with 4 µg of Bam Hl-digested Ptk-1 DNA along with 4 µg of Hind III-cleaved plasmid pHβ-8 containing the chromosomal adult human β-globin gene, Lawn, R. M., et al., Cell 15:1157-1174 (1978). Tk^+ transformants were selected in growth medium containing 0.1 mM hypoxanthine/0.4 µM aminopterin/16 µM thymidine (HAT). Colonies were picked with cloning cylinders and were grown into mass cultures.

Cotransformation of Defined DNA Sequences and the HSV tk Gene

Ltk^- $aprt^-$ mouse cells were transformed with either 1 - 10 µg of ΦX174, 1 µg of pBR322 or 1 µg of RβG-1 DNA in the presence of 1 ng of HSV-1 tk gene and 10-20 µg of salmon sperm carrier DNA, as previously described. Wigler, M. et al., PNAS 76:1373-1376 (1979) . Tk^+ transformants were selected in DME containing hypoxanthine, aminopterin and thymidine (HAT) and 10% calf serum. Isolated colonies were picked using cloning cylinders and grown into mass cultures.

Enzyme Assays

Extracts were prepared by resuspending washed cell pellets

(approximately 10^7 cells) in 0.1 ml of 0.02 M potassium phosphate, pH 7, containing 0.5% Triton X-100. The supernatant (cytoplasm) obtained after 25 min of 700 X g centrifugation was used for the quantitation of enzymatic activity and for electrophoresis. aprt and protein were assayed as previously described. Chasin, L. A., Cell 2:37-41 (1974). Inclusion of 3 mM thymidine triphosphate, an inhibitor of 5'-nucleotidase, Murray, A. W. and Friedrichs, B., Biochem, J. 111:83-89 (1969), in the reaction mixture did not increase AMP recovery, indicating that the nucleotidase was not interfering with the measurement of aprt activity. Isoelectric focusing of aprt was carried out essentially as described for hypoxanthine phosphoribosyltransferase, Chasin, L. A. and Urlaub, G. Somat. Cell Genet. 2:453-467 (1976), with the following exceptions: The polyacrylamide gel contained an Ampholine (LKB) mixture of 0.8% pH 2.5-4, 0.8% pH 4-6, and 0.4% pH 5-7. For assaying enzymatic activity, [2-^{3}H] adenine [0.04 mM, 1 Ci/mmol, New England Nuclear (1 Ci = 3.7 X 10^{10} becquerels)] was substituted for hypoxanthine.

Assays of Thymidine Kinase Activity

For specific activity measurements, cells from monolayer cultures were scraped into phosphate buffered saline and washed. The cell pellet was suspended in 5 volumes of extraction buffer (0.01 M Tris·HCl, pH 7.5, 0.01 M KCl, 1mM $MgCl_2$, 1mM 2-mercaptoethanol, and 50 μM thymidine). The cell suspension was frozen and thawed three times and the KCl concentration was then adjusted to 0.15 M. After sonication, the cytoplasmic extract was obtained by centrifugation at 30,000 X g for 30 min, and the supernatant was used for tk assays as described in Wigler, M. et al. Cell 16:777-785 (1979). Cytoplasmic extracts from tumors were obtained after disruption of the cells in a Potter-Elvejehm homogenizer. They were then treated as described above for cultured cells. One unit of thymidine kinase is defined as the amount of enzyme which converts one nanomole of thymi-

dine into tnymidine monophosphate per minute.

In enzyme neutralization studies, anti-HSV-1 tk antiserum or preimmune serum was mixed with an equal volume of cytoplasmic extract, and ATP and magnesium were added to 6.7 mM. The enzyme-antibody mixture was incubated for 30 min at room temperature, centrifuged at 2,000 X g for 10 min, and the supernatant was assayed for tk activity.

In an additional biochemical assay, 30,000 X g supernatants of homogenates from cell cultures and from solid tumors were electrophoresed on 5% polyacrylamide gels which were then cut into 1.6 mm slices and assayed for tk activity as described. Lee, L. S. and Cheng, Y. C., J. Biol. Chem., 251:2600-2604 (1976).

RNA Isolation

Total RNA was isolated from logarithmic-phase cultures of transformed L cells by successive extractions with phenol at pH 5.1, phenol/chloroform/isoamyl alcohol (25:24:1, vol/vol), and chloroform/isoamyl alcohol (24:1, vol/vol). After ethanol precipitation, the RNA was digested with DNase, Maxwell, I. H., et al., Nucleic Acids Res. 4:241-246 (1977) and precipitated with ethanol. Nuclear and cytoplasmic fractions were isolated as described in Wigler, M. et al., PNAS 76:1373-1376 (1979) and RNAs were extracted as described above. Cytoplasmic polyadenylylated RNA was isolated by oligo(dT)-cellulose chromatography. Axel, R. et al., Cell 7:247-254 (1976).

cDNA Synthesis

Rabbit and mouse cDNAs were prepared by using avian myeloblastosis virus reverse transcriptase (RNA-dependent DNA polymerase) as described in Myers, J. C. and Spiegelman, S., PNAS 75:5329-5333 (1978).

Isolation of Transformed Cell DNA

Cells were harvested by scraping into PBS and centrifuging at 1000 X g for 10 min. The pellet was resuspended in 40 vol of TNE [10 mM Tris-HCl (ph 8.0), 150 mM NaCl, 10 mM EDTA], and SDS and proteinase K were added to 0.2% and 100 µg/ml, respectively. The lysate was incubated at 37°C for 5-10 hr and then extracted sequentially with buffer-saturated phenol and $CHCl_3$. High molecular weight DNA isolated by mixing the aqueous phase with 2 vol of cold ethanol and immediately removing the precipitate that formed. The DNA was washed with 70% ethanol and dissolved in 1 mM Tris, 0.1 EDTA.

Nuclei and cytoplasm from clones ϕX4 and ϕX5 were prepared as described by Ringold, G. M., et al. Cell 10:19-26 (1977). The nuclear fraction was further fractionated into high and low molecular weight DNA as described by Hirt, B., J. Mol. Biol. 26:365-369 (1967).

DNA Filter Hybridizations

Cellular DNA was digested with restriction endonucleases, electrophoresed on agarose slab gels, transferred to nitrocellulose filter sheets, and hybridized with ^{32}P-labeled DNA probes as described by Wigler, M. et al., PNAS 76:1373-1376 (1979).

DNA from transformed cells was digested with various restriction endonucleases using the conditions specified by the supplier (New England Biolabs or Bethesda Research Laboratories). Digestions were performed at an enzyme to DNA ratio of 1.5 U/µg for 2 hr at 37°C. Reactions were terminated by the addition of EDTA, and the product was electrophoresed on horizontal agarose slab gels in 36 mM

Tris, 30 mM NaH_2PO_4, 1 mM EDTA (pH 7.7). DNA fragments were transferred to nitrocellulose sheets, hybridized and washed as previously described. Weinstock, R., et al., PNAS 75:1299-1303 (1978) with two modifications. Two nitrocellulose filters were used during transfer. Jeffreys, A. J. and Flavell, R. A., Cell 12:1097-1108 (1977). The lower filter was discarded, and following hybridization the filter was washed 4 times for 20 min in 2 X SSC, 25 mM sodium phosphate, 1.5 mM $Na_4P_2O_7$, 0.05% SDS at 65°C and then successively in 1:1 and 1:5 dilutions of this buffer. Jeffreys, A. J. and Flavell, R. A., Cell 12:429-439 (1977).

In the amplification experiments the probes were either ^{32}P-nick translated pBR322 or pdhfr-21, a cDNA copy of mouse dhfr mRNA. Chang, A.C.Y., et al., Nature 275:617-624 (1978).

Solution Hybridizations

^{32}P-Labeled globin cDNAs (specific activities of 2-9 X 10^8 cpm/μg) were hybridized with excess RNA in 0.4 M NaCl/25 mM 1,4-piperazinediethanesulfonic acid (Pipes), pH 6.5/5 mM EDTA at 75°C. Incubation times did not exceed 70 hr. R_0ts were calculated as moles of RNA nucleotides per liter times time in seconds. The fraction of cDNA rendered resistant to the single-strand nuclease S1 in hybridization was determined as described. Axel, R. et al., Cell 7:247-254 (1976).

RNA Filter Hybridization

RNA was electrophoresed through 1% agarose slab gels (17 X 20 X 0.4 cm) containing 5 mM methylmercury hydroxide as described by Bailey, J. and Davidson, N., Anal. Biochem.

70:75-85 (1976). The concentration of RNA in each slot was 0.5 µg/µl. Electrophoresis was at 110 V for 12 hr at room temperature.

RNA was transferred from the gel to diazotized cellulose paper as described by Alwine, J. C. , et al., PNAS 74:5350-5354 (1979) by using pH 4.0 citrate transfer buffer. After transfer, the RNA filter was incubated for 1 hr with transfer buffer containing carrier RNA at 500 µg/ml. The RNA on the filters was hybridized with cloned DNA probe at 50 ng/ml labeled by ^{32}P-nick translation, Weinstock, R., et al., PNAS 75:1299-1303 (1978) to specific activities of 2-8 X 10^8 cpm/µg. Reaction volumes were 25 µl/cm^2 of filter. Hybridization was in 4X standard saline citrate (0.15 M NaCl/0.015 M sodium citrate)/50% formamide at 57°C for 36-48 hr.

After hybridization, filters were soaked in two changes of 2X standard saline citrate/25 mM sodium phosphate/1.5 mM sodium pyrophosphate/0.1% sodium dodecyl sulfate/5 mM EDTA at 37°C for 30 min with shaking to remove formamide. Successive washes were at 68°C with 1X and 0.1X standard saline citrate containing 5 mM EDTA and 0.1% sodium dodecyl sulfate for 30 min each.

Berk Sharp Analysis of Rabbit ß-Globin RNA in Transformed Mouse L Cells

The hybridizations were carried out in 80% (vol/vol) formamide (Eastman)/0.4 M Pipes, pH 6.5/0.1 mM EDTA/0.4 M NaCl, Casey, J. and Davidson, N., Nucleic Acid Res., 4:1539-1552 (1977); Berk, A. J. and Sharp, P. A., Cell 12: 721-732 (1977) for 18 hr at 51°C for the 1.8 kbp Hha I fragment and 49°C for the Pst 1 fragment. The hybrids were treated with S1 nuclease and analyzed essentially by the procedure described by Berk, A. J. and Sharp, P. A. (1977).

Although the instant disclosure sets forth all essential information in connection with the invention, the numerous publications cited herein may be of assistance in understanding the background of the invention and the state of the art. Accordingly, all of the publications cited are hereby incorporated by reference into the present disclosure.

Can Claim is claimed as;

Claim 1

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WHAT IS CLAIMED IS:

1. A process for inserting foreign DNA I into a eucaryotic cell which comprises cotransforming said eucaryotic cell with said foreign DNA I and with unlinked foreign DNA II which codes for a selectable phenotype not expressed by said eucaryotic cell, said cotransformation being carried out in a suitable medium and in the presence of selective conditions permitting survival or identification of eucaryotic cells which have acquired said selectable phenotype.

2. A process in accordance with Claim 1 wherein said foreign DNA I codes for proteinaceous material which is not associated with a selectable phenotype.

3. A process in accordance with Claim 2 wherein said foreign DNA I codes for interferon protein.

4. A process in accordance with Claim 2 wherein said foreign DNA I codes for insulin.

5. A process in accordance with Claim 2 wherein said foreign DNA I codes for growth hormone.

6. A process in accordance with Claim 2 wherein said foreign DNA I codes for a clotting factor.

7. A process in accordance with Claim 2 wherein said foreign DNA I codes for a viral antigen or an antibody.

8. A process in accordance with Claim 2 wherein said foreign DNA I codes for an enzyme.

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9. A process in accordance with Claim 1 wherein said foreign DNA is substantially purified.

10. A process in accordance with Claim 1 wherein said foreign DNA I and/or DNA II are attached to bacterial plasmid or phage DNA.

11. A process in accordance with Claim 1 wherein said foreign DNA I and/or DNA II are attached to phage DNA encapsidated in a phage particle.

~~12.~~ 10. A process in accordance with Claim 1 wherein said foreign DNA I has been obtained from restriction endonuclease cleavage of eucaryotic chromosomal DNA.

~~13.~~ 11. A process in accordance with Claim 1 wherein said foreign DNA I and DNA II have been treated with calcium phosphate.

~~14.~~ 12. A process in accordance with Claim 1 wherein said eucaryotic cell is a mammalian cell.

~~15.~~ 13. A process in accordance with Claim ~~14~~ 12 wherein said mammalian cell is an erythroblast.

~~16.~~ 14. A process in accordance with Claim ~~14~~ 12 wherein said mammalian cell is a fibroblast.

~~17.~~ 15. A process in accordance with Claim 1 wherein said foreign DNA I is present in an amount relative to said DNA II which codes for a selectable phenotype in the range from about 1:1 to about 100,000:1.

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~~18.~~ 16. A process in accordance with Claim 1 wherein said DNA II which codes for a selectable phenotype comprises the gene for thymidine kinase from herpes simplex virus.

~~19.~~ 17. A process in accordance with Claim 1 wherein said DNA II which codes for proteinaceous material which is associated with a selectable phenotype comprises the gene for adenine phosphoribosyl-transferase.

~~20.~~ 18. A process in accordance with Claim 1 wherein said DNA II which codes for a selectable phenotype comprises a gene associated with drug resistance.

~~21.~~ 19. A process in accordance with Claim ~~20~~ 18 wherein said gene associated with drug resistance is the gene coding for a mutant dihydrofolate reductase which renders cells resistant to methotrexate.

22. A process in accordance with Claim 1 wherein said foreign DNA I is incorporated into the chromosomal DNA of said eucaryotic cell.

23. A process for inserting purified foeign DNA I coding for proteinaceous material which is not associated with a selectable phenotype into a eucaryotic cell which comprises cotransforming said eucaryotic cell with said foreign DNA I coding for proteinaceous material which is not associated with a selectable phenotype and with physically unlinked foreign DNA II coding for proteinaceous material which is associated with a selectable phenotype, said cotransformation being carried out in the presence of selective conditions permitting survival or identification of eucaryotic cells which have acquired said selectable phenotype.

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~~24.~~ A process in accordance with Claim ~~23~~ 28 wherein said proteinaceous material which is not associated with a selectable phenotype comprises interferon protein, insulin, growth hormone, clotting factor, viral antigen or antibody.

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~~25.~~ A eucaryotic cell into which foreign DNA I has been inserted in accordance with the process of Claim 1.

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~~26.~~ A mammalian cell into which foreign DNA I has been inserted in accordance with the process of Claim 1.

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~~27.~~ A eucaryotic cell into which foreign DNA I has been inserted in accordance with the process of Claim ~~28~~ 28.

28. A eucaryotic cell which comprises foreign DNA I coding for proteinaceous material which is not associated with a selectable phenotype.

29. A mammalian cell in accordance with Claim 28.

30. A eucaryotic cell in accordance with Claim 28 wherein said proteinaceous material which is not associated with a selectable phenotype comprises interferon protein, antibody, insulin, growth hormone, clotting factor or viral antigen.

Claim 22

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31. A process for producing proteinaceous material which comprises cotransforming a eucaryotic cell with foreign DNA I coding for said proteinaceous material in accordance with the process of Claim 1, maintaining said cotransformed eucaryotic cell under suitable conditions to enable said foreign DNA I to be transcribed to form complementary RNA and said complementary RNA so formed to be translated to produce said proteinaceous material, and recovering said proteinaceous material so produced.

32. A process in accordance with Claim 31 wherein said proteinaceous material comprises interferon protein, insulin, growth hormone, clotting factor, viral antigen or antibody.

33. A process in accordance with Claim 31 wherein said eucaryotic cell is a mammalian cell.

34. A process in accordance with Claim 31 wherein said foreign DNA I coding for said proteinaceous material is purified DNA.

35. A process for producing proteinaceous material which comprises cotransforming a eucaryotic cell with foreign DNA I coding for said proteinaceous material in accordance with the process of Claim 1, culturing or cloning said selective conditions to produce a multiplicity of eucaryotic cells derived therefrom and recovering said proteinaceous material from the eucaryotic cells so produced.

~~36.~~ A process in accordance with Claim ~~35~~ wherein said proteinaceous material comprises interferon protein, insulin, growth hormone, clotting factor, viral antigen or antibody.

~~37.~~ A process in accordance with Claim ~~35~~ wherein said eucaryotic cell is a mammalian cell.

38. A process in accordance with Claim 35 wherein said foreign DNA I is purified DNA.

39. A process for producing proteinaceous material which is not associated with a selectable phenotype which comprises cotransforming a eucaryotic cell with foreign DNA I coding for said proteinaceous material in accordance with the process of Claim 23, culturing or cloning said cotransformed eucaryotic cell in the presence of said selective conditions to produce a multiplicity of eucaryotic cells derived therefrom, and recovering said proteinaceous material from the eucaryotic cells so produced.

40. A process in accordance with Claim 39 wherein said proteinaceous material comprises interferon protein, insulin, growth hormone, clotting factor, viral antigen or antibody.

41. A process in accordance with Claim 39 wherein said eucaryotic cell is a mammalian cell.

42. A process in accordance with Claim 39 wherein said foreign DNA is purified DNA.

43. Proteinaceous material produced in accordance with the process of Claim 35.

44. Proteinaceous material produced in accordance with the process of Claim 39.

45. A process for inserting a multiplicity of foreign DNA I molecules corresponding to multiple copies of a gene coding for a proteinaceous material into a eucaryotic cell which comprises cotransforming said eucaryotic cell with said multiplicity of foreign DNA I molecules and with a multiplicity of unlinked DNA II molecules coding for a selectable phenotype not expressed by said eucaryotic cell, said cotransformation being carried out in a suitable medium and in the presence of an agent permitting survival or identification of eucaryotic cells which have acquired said multiplicity of genes coding for said selectable phenotype.

32 ~~46.~~ A process in accordance with Claim ~~45~~ 31 wherein said foreign DNA I codes for proteinaceous material which is not associated with a selectable phenotype.

33 ~~47.~~ A process in accordance with Claim ~~46~~ 32 wherein said foreign DNA I codes for interferon protein.

34 ~~48.~~ A process in accordance with Claim ~~46~~ 32 wherein said foreign DNA I codes for insulin.

35 ~~49.~~ A process in accordance with Claim ~~46~~ 32 wherein said foreign DNA I codes for growth hormone.

36 ~~50.~~ A process in accordance with Claim ~~46~~ 32 wherein said foreign DNA I codes for a clotting factor.

37 ~~51.~~ A process in accordance with Claim ~~46~~ 32 wherein said foreign DNA I codes for a viral antigen or an antibody.

38 ~~52.~~ 32 A process in accordance with Claim ~~46~~ wherein said foreign DNA I codes for an enzyme.

39 ~~53.~~ 31 A process in accordance with Claim ~~45~~ wherein said foreign DNA I is substantially purified.

40 ~~54.~~ 31 A process in accordance with Claim ~~45~~ wherein said foreign DNA I has been obtained from restriction endonuclease cleavage of eucaryotic chromosomal DNA.

55. A process in accordance with Claim 45 wherein said foreign DNA I and/or DNA II are attached to bacterial or phage DNA.

56. A process in accordance with Claim 45 wherein said foreign DNA I and/or DNA II are attached to phage DNA encapsidated in a phage particle.

41 ~~57.~~ 31 A process in accordance with Claim ~~45~~ wherein said foreign DNA I and DNA II have been treated with calcium phosphate.

42 ~~58.~~ 31 A process in accordance with Claim ~~45~~ wherein said eucaryotic cell is a mammalian cell.

43 ~~59.~~ 42 A process in accordance with Claim ~~58~~ wherein said mammalian cell is an erythroblast.

44 ~~60.~~ 42 A process in accordance with Claim ~~58~~ wherein said mammalian cell is a fibroblast.

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45 ~~61.~~ A process in accordance with Claim ~~45~~ 31 wherein said foreign DNA I is present in an amount relative to said DNA II which codes for proteinaceous material associated with a selectable phenotype in the range from about 1:1 to about 100,000:1.

46 ~~62.~~ A process in accordance with Claim ~~45~~ 31 wherein said foreign DNA II which codes for proteinaceous material which is associated with a selectable phenotype comprises a gene associated with drug resistance.

47 ~~63.~~ A process in accordance with Claim ~~62~~ 46 wherein said gene associated with drug resistance is a gene coding for a mutant dihydrofolate reductase which renders cells resistant to methotrexate.

48 ~~64.~~ A process in accordance with Claim ~~45~~ 31 wherein said foreign DNA I is incorporated into the chromosomal DNA of said eucaryotic cell.

49 ~~65.~~ A eucaryotic cell into which foreign DNA I has been inserted in accordance with the process of Claim ~~45~~ 31.

50 ~~66.~~ A mammalian cell into which foreign DNA I has been inserted in accordance with the process of Claim ~~45~~ 31.

67. A eucaryotic cell which comprises a multiplicity of foreign DNA I molecules.

68. A eucaryotic cell in accordance with Claim 67 which comprises a multiplicity of foreign DNA I molecules coding for proteinaceous material which is not associated with a selectable phenotype.

69. A eucaryotic cell in accordance with Claim 68 wherein said proteinaceous material which is not associated with a selectable phenotype comprises interferon protein, insulin, growth hormone, clotting factor, viral antigen or antibody.

70. A mammalian cell in accordance with Claim 67.

71. A process for producing proteinaceous material which comprises cotransforming a eucaryotic cell with a multiplicity of foreign DNA I molecules coding for said proteinaceous material in accordance wtih the process of Claim 45, maintaining said cotransformed eucaryotic cell under suitable conditions to enable said foreign DNA I to be transcribed to form complementary RNA and said complementary RNA so formed to be translated to produce said proteinaceous material, and recovering said proteinaceous material so produced.

72. A process in accordance with claim 71 wherein said proteinaceous material comprises interferon protein, insulin, growth hormone, clotting factor, viral antigen or antibody.

73. A process in accordance with claim 71 wherein said eucaryotic cell is a mammalian cell.

74. A process in accordance with Claim 71 wherein said foreign DNA I coding for said proteinaceous material is purified DNA.

75. A process for producing proteinaceous material which comprises cotransforming a eucaryotic cell with a multiplicity of foreign DNA I molecules coding for said proteinaceous material in accordance with the process of Claim 45, culturing or cloning said cotransformed eucaryotic cell in the presence of said agent permitting survival or identification of eucaryotic cells which have acquired said multiplicity of genes coding for said selectable phenotype to produce a multiplicity of eucaryotic cells derived therefrom, and recovering said proteinaceous material from the eucaryotic cells so produced.

76. A process in accordance with Claim 75 wherein said proteinaceous material comprises interferon protein, insulin, growth hormone, clotting factor, viral antigen or antibody.

77. A process in accordance with Claim 75 wherein said eucarotic cell is a mammalian cell.

78. A process in accordance with Claim 75 wherein said foreign DNA I is purified DNA.

79. Proteinaceous material produced in accordance with Claim 71.

80. Proteinaceous material produced in accordance with Claim 75.

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~~81.~~ A process for generating a multiplicity of foreign DNA I molecules corresponding to multiple copies of a gene in a eucaryotic cell which comprises transforming said eucaryotic cell with a molecule which is formed by linking one of said foreign DNA I molecules to a DNA II molecule corresponding to an amplifiable gene for a dominant selectable phenotype not expressed by said eucaryotic cell, and culturing the transformed eucaryotic cells in the presence of successively elevated concentrations of an agent permitting survival or identification of eucaryotic cells which have acquired multiple copies of said amplifiable gene, said transformation and culturing being carried out ~~in a suitable medium.~~ under suitable conditions

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~~82.~~ A process in accordance with Claim ~~81~~ 54 wherein said foreign DNA I codes for proteinaceous material which is not associated with a selectable phenotype.

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~~83.~~ A process in accordance with Claim ~~82~~ 55 wherein said foreign DNA I codes for interferon protein.

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~~84.~~ A process in accordance with Claim ~~82~~ 55 wherein said foreign DNA I codes for insulin.

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~~85.~~ A process in accordance with Claim ~~82~~ 55 wherein said foreign DNA I codes for growth hormone.

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~~86.~~ A process in accordance with Claim ~~82~~ 55 wherein said foreign DNA I codes for a clotting factor.

60. ~~87.~~ A process in accordance with Claim ~~82~~ 55 wherein said foreign DNA I codes for a viral antigen or antibody.

61. ~~88.~~ A process in accordance with Claim ~~82~~ 55 wherein said foreign DNA I codes for an enzyme.

62. ~~89.~~ A process in accordance with Claim ~~81~~ 54 wherein said foreign DNA I is substantially purified.

63. ~~90.~~ A process in accordance with Claim ~~81~~ 54 wherein said foreign DNA I has been obtained from restriction endonuclease cleavage of eucaryotic chromosomal DNA.

91. A process in accordance with Claim 81 wherein said foreign DNA I and/or DNA II are attached to bacterial or phage DNA.

92. A process in accordance with Claim 81 wherein said foreign DNA I and/or DNA II are attached to phage DNA encapsidated in a phage particle.

64. ~~93.~~ A process in accordance with Claim ~~81~~ 54 wherein said foreign DNA I and DNA II have been treated with calcium phosphate.

65. ~~94.~~ A process in accordance with Claim ~~81~~ 54 wherein said eucaryotic cell is a mammalian cell.

66. ~~95.~~ A process in accordance with Claim ~~94~~ 65 wherein said mammalian cell is an erythroblast.

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67 ~~96.~~ A process in accordance with Claim 65 ~~94~~ wherein said mammalian cell is a fibroblast.

68 ~~97.~~ A process in accordance with Claim 54 ~~81~~ wherein said foreign DNA I is present in an amount relative to said DNA II which codes for proteinaceous material associated with a selectable phenotype in the range from about 1:1 to about 100,000:1.

69 ~~98.~~ A process in accordance with Claim 54 ~~81~~ wherein said DNA II which codes for proteinaceous material which is associated with a selectable phenotype comprises a gene associated with resistance to a drug or chemical antagonist.

70 ~~99.~~ A process in accordance with Claim 69 ~~98~~ wherein said gene associated with resistance to a drug or chemical antagonist is a gene coding for a mutant dihydrofolate reductase which renders cells resistant to methotrexate.

71 ~~100.~~ A process in accordance with Claim 54 ~~81~~ wherein said foreign DNA I is incorporated into the chromosomal DNA of said eucaryotic cell.

72 ~~101.~~ A eucaryotic cell into which foreign DNA I has been inserted in accordance with the process of Claim 54 ~~81~~.

73 ~~102.~~ A mammalian cell into which foreign DNA I has been inserted in accordance with the process of Claim 54 ~~81~~.

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103. A process for producing proteinaceous material which comprises transforming a eucaryotic cell with a multiplicity of foreign DNA I molecules coding for proteinaceous material in accordance with the process of Claim 81, maintaining said transformed eucaryotic cells under suitable conditions to enable said multiplicity of foreign DNA I genes to be transcribed to form complementary RNA and said complementary RNA so formed to be translated to produce said proteinaceous material, and recovering said proteinaceous material so produced.

104. A process in accordance with Claim 103 wherein said proteinaceous material comprises interferon protein, insulin, growth hormone, clotting factor, viral antigen or an antibody.

105. A process in accordance with Claim 103 wherein said eucaryotic cell is a mammalian cell.

106. A process in accordance with Claim 103 wherein said foreign DNA I coding for said proteinaceous material is purified DNA.

107. A process for producing proteinaceous material which comprises transforming a eucaryotic cell with a multiplicity of foreign DNA I molecules coding for said proteinaceous material in accordance with the process of Claim 81, culturing or cloning said transformed eucaryotic cells in the presence of said agent permitting survival or identification of eucaryotic cells which have acquired said dominant selectable phenotype so as to produce a multiplicity of eucaryotic cells derived therefrom, and recovering said proteinaceous material from the eucaryotic cells so produced.

108. A process in accordance with Claim 107 wherein said proteinaceous material comprises interferon protein, insulin, growth hormone, clotting factor, viral antigen or an antibody.

109. A process in accordance with Claim 107 wherein said eucaryotic cell is a mammalian cell.

110. A process in accordance with Claim 107 wherein said foreign DNA I is purified DNA.

111. Proteinaceous material produced in accordance with Claim 103.

112. Proteinaceous material produced in accordance with Claim 107.

113. A process for cotransforming a eucaryotic cell which comprises transforming said eucaryotic cell with foreign DNA I and with DNA II, said DNA I and DNA II being unlinked and said DNA II coding for a selectable phenotype not expressed by said eucaryotic cell prior to cotransformation.

114. A method of detecting eucaryotic cells which have been transformed with foreign DNA I which is not associated with a selectable phenotype which comprises cotransforming said eucaryotic cell with said DNA I and with DNA II which is associated with a selectable phenotype in accordance with the process of Claim 1, and screening for eucaryotic cells so co-transformed.

115. A process for producing a biological material, a portion of which is proteinaceous, which comprises producing said proteinaceous portion within a eucaryotic cell in accordance with any of the processes of Claims 31, 35, 39, 71, 75, ~~103 or 107~~, maintaining said eucaryotic cell under suitable conditions to permit the eucaryotic cell to form, synthesize, or assemble said material, and recovering said material so produced.

116. A process in accordance with Claim 115 wherein said compound is interferon.

117. Biological material produced in accordance with Claim 115.

~~118.~~ A process for inserting ~~foreign~~ foreign DNA I into a eucaryotic cell which comprises cotransforming said eucaryotic cell with said foreign DNA I and with unlinked foreign DNA II which codes for a selectable phenotype not expressed by said eucaryotic cell, said cotransformation being carried out in a suitable medium and in the presence of conditions permitting identification and recovery of eucaryotic cells which have acquired said selectable phenotype.

119. A process for producing a non-proteinaceous biological material, the synthesis or assembly of which is regulated or otherwise controlled by a gene, which comprises cotransforming a eucaryotic cell with said gene and with a gene coding for a selectable phenotype, maintaining said eucaryotic cell under suitable conditions to permit said eucaryotic cell to synthesize or assemble said non-proteinaceous biological material, and recovering said biological material so produced.

120. A pharmaceutical composition which comprises a pharmaceutically effective amount of a proteinaceous material produced in accordance with any of the processes of Claims 31, 35, 39, 71, 75, 103 or 107 and a physiologically acceptable carrier.

121. A pharmaceutical composition which comprises a pharmaceutically effective amount of a biological material in accordance with Claim 117 and a physiologically acceptable carrier.

122. A pharmaceutical composition which comprises a pharmaceutically effective amount of a non-proteinaceous material produced in accordance with Claim 119 and a physiologically acceptable carrier.

123. A method of treatment which comprises administering to a patient a pharmaceutical composition in accordance with any of Claims 120, 121 or 122.

124. A method for recovering a desired eucaryotic gene not subject to cleavage by endonuclease I from a eucaryotic cell X, which includes said desired gene which comprises transforming a eucaryotic cell Y deficient in said eucaryotic gene with hybrid-DNA, said hybrid-DNA being formed by ligating total eucaryotic cell X DNA cleaved by endonuclease I with plasmid DNA cleaved with endonuclease I, so as to effect integration of said hybrid-DNA into said eucaryotic cell Y DNA, selecting for eucaryotic cells Y containing said desired eucaryotic gene, removing said eucaryotic cell Y DNA containing said desired eucaryotic gene, cleaving said removed eucaryotic cell Y DNA with an endonuclease which does not cleave either said plasmid DNA or said desired

eucaryotic gene, circularizing the resulting cleaved DNA fragments with ligase, transforming a bacterial cell with said circularized DNA fragments so as to produce transformed bacterial cells containing said desired eucaryotic gene and said plasmid DNA and removing said desired gene therefrom.

125. A method for recovering a desired eucaryotic gene not subject to cleavage by endonuclease I from a eucaryotic cell X which includes said desired gene which comprises cotransforming a eucaryotic cell Y deficient in said eucaryotic gene with total eucaryotic cell X DNA cleaved by endonuclease I and with plasmid DNA so as to effect integration of said desired eucaryotic gene into said eucaryotic cell Y DNA, selecting for eucaryotic cells Y containing said desired eucaryotic gene and said plasmid DNA, removing said eucaryotic cell Y DNA containing said desired eucaryotic gene, cleaving said removed eucaryotic cell Y DNA with an endonuclease which does not cleave either said plasmid DNA or said desired eucaryotic gene, circularizing the resulting cleaved DNA fragments with ligase, transforming a bacterial cell with said circularized DNA fragments so as to produce transformed bacterial cells containing said desired eucaryotic gene and said plasmid DNA and recovering said desired gene therefrom.

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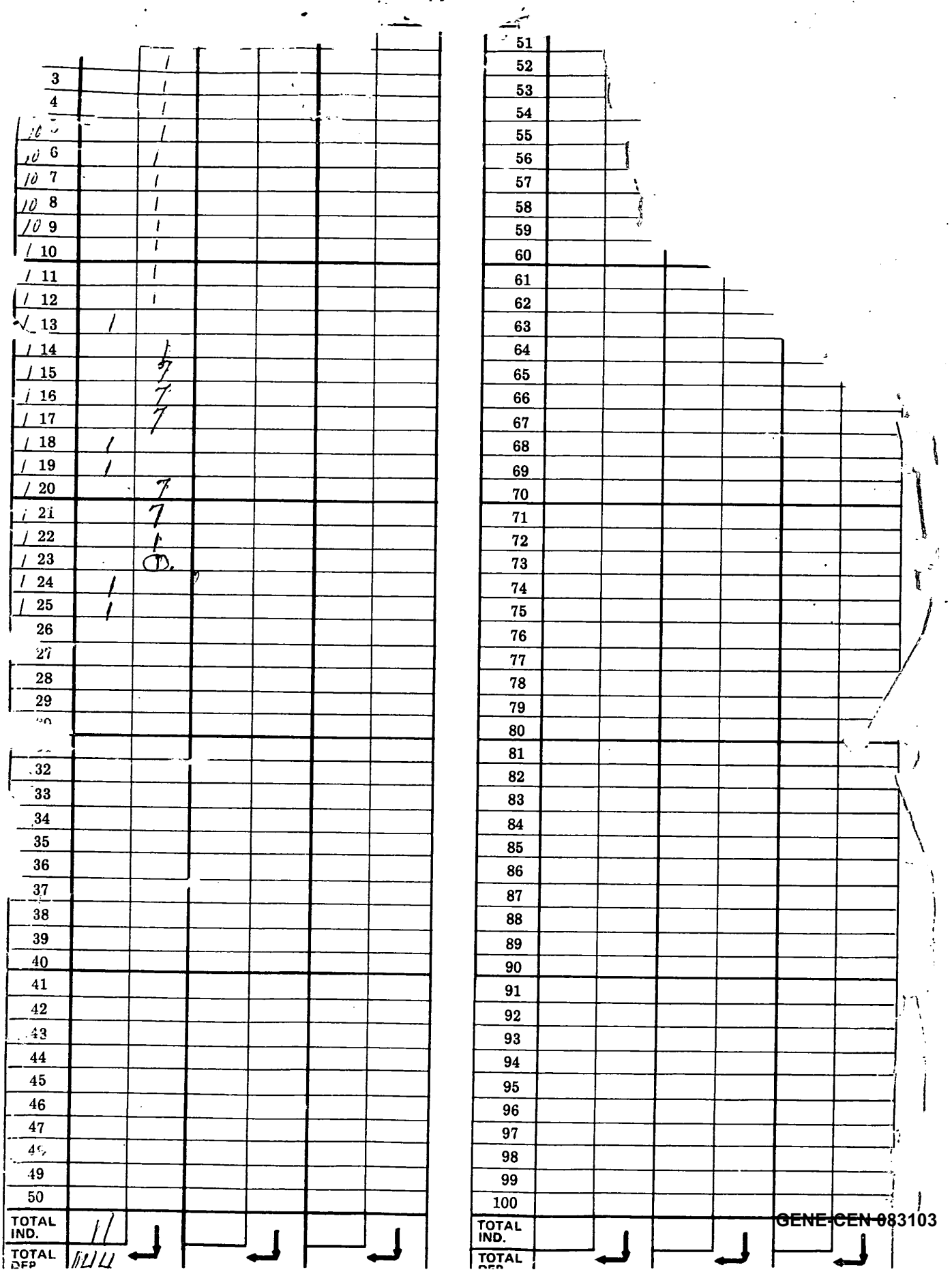

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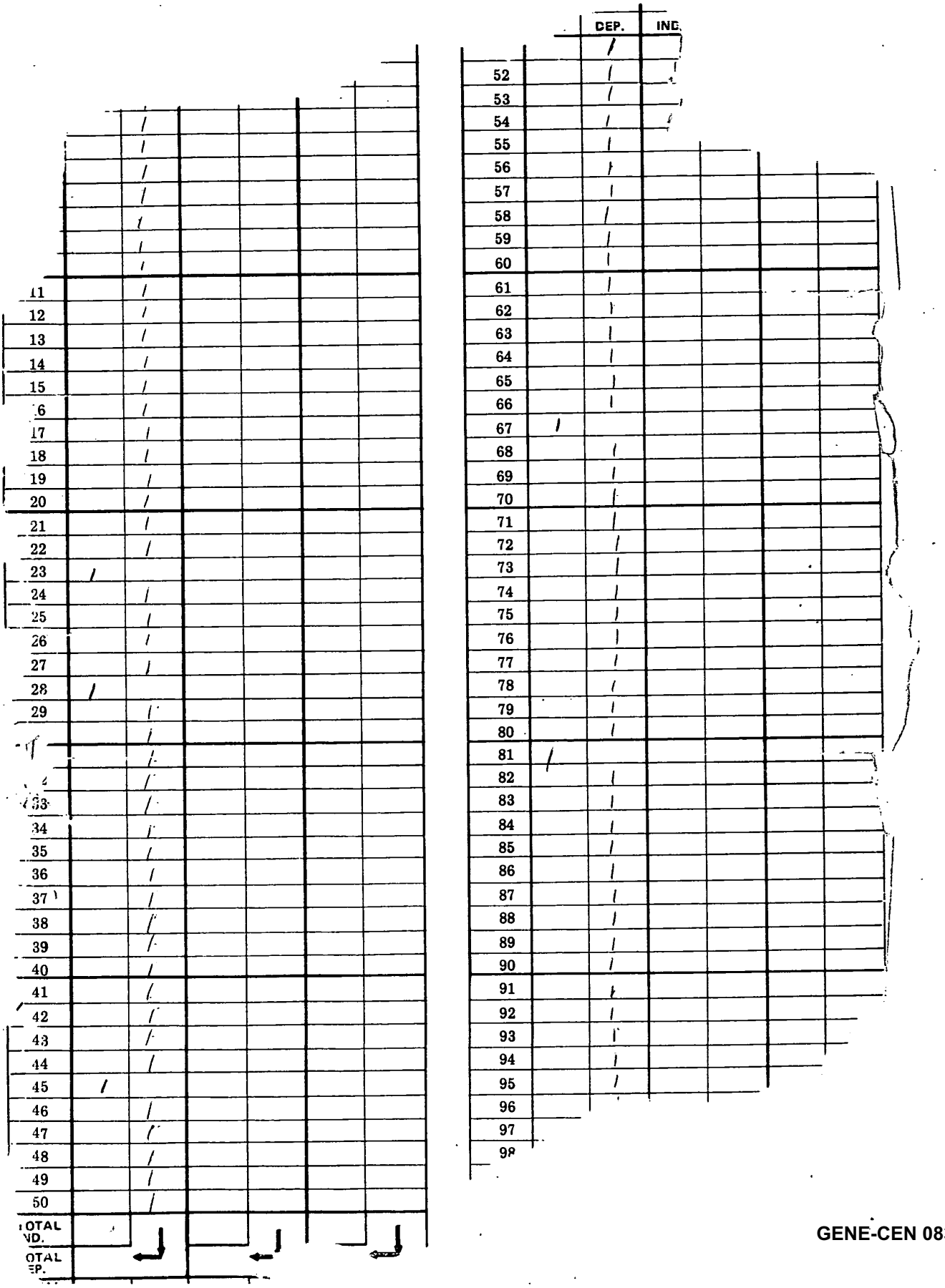

GENE-CEN 083104

SUBCLAS 6

GROUP ART UNIT 125 174

Art/435

L H. WIGLER, COLD SPRING HAR, NY;

4399216

DATA

APPLICATIONS

[redacted]

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446-47-6-3
#61

Small Entity

Foreign priority claimed: 35 USC 119 conditions met / Verified and Acknowledged	yes ☑ no / yes ☑ no / Examiner's initials	AS FILED	STATE OR COUNTRY	SHEETS DRWGS.	TOTAL CLAIMS	INDEP. CLAIMS	FILING FEE RECEIVED	ATTORNEY'S DOCKET NO.
			NY	2	125	11	$ 455	

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PROCESSES FOR INSERTING DNA INTO EUCARYOTIC CELLS AND FOR PRODUCING PROTEINACEOUS MATERIALS

U.S. DEPT. of COMM.-Pat. & TM Office — PTO-436L (rev.

CERTIFICATE
MAR 26 1985
OF CORRECTION

(2)

PARTS OF APPLICATION FILED SEPARATELY

PREPARED FOR ISSUE

(Assistant Examiner) E.G. Hot (Docket Clerk)

AT ALLOWANCE

FIGURES DRWGS.	CLAIMS	CLASS	SUBCLASS
2	73	435	6

EXAMINED AND PASSED FOR ISSUE

ESTHER M. KEPPLINGER
PRIMARY EXAMINER
(Primary Examiner) 172 (Art Unit)

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

GROUP 170

Applicant : Richard Axel, et al.

Serial No.: 124,513 Examiner: Esther Kepplinger

Filed : February 25, 1980 Group Art Unit: 170

For : PROCESSES FOR INSERTING DNA INTO EUCARYOTIC CELLS AND FOR PRODUCING PROTEINACEOUS MATERIALS

30 Rockefeller Plaza
New York, New York 10112

1 May 1981

Honorable Commissioner of Patents
and Trademarks
Washington, D.C. 20231

SIR:

COMMUNICATION

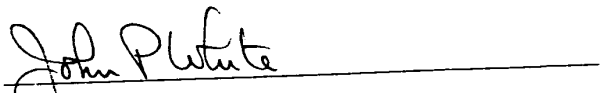
In response to the Examiner's telephone request, applicants herewith submit copies of the following articles:

EXHIBIT	REFERENCE
A	Kretschmer, P.J., et al., "Indirect Selection of Bacterial Plasmids Lacking Identifiable Phenotypic Properties," J. Bacteriology 124: 225-231 (1975);
B	Szybalska, E.H. and Szybalski, W., "Genetics of Human Cell Lines, IV. DNA-Mediated Heritable Transformation of a Biochemical Trait," PNAS 48: 2026-2034 (1962);
C	McCutchan, J.H. and Pagano, J.S., "Enhancement of the Infectivity of Simian Virus 40 Deoxyribonucleic Acid with Diethylaminoethyl-Dextran," Journal National Cancer Institute, 41: 351-356 (1968);
D	Wigler, M., et al., "Transfer of Purified Herpes Virus Thymidine Kinase Gene to Cultured Mouse Cells," Cell 11: 223-232 (1977);

EXHIBIT	REFERENCE
E	Wigler, M., et al., "Transformation of Mammalian Cells with Genes from Procaryotes and Eucaryotes," Cell 16: 777-785 (1979);
F	Wold B., et al., "Introduction and Expression of a Rabbit β-globin Gene in Mouse Fibroblasts," PNAS 76: 5684-5688 (1979);
G	Wigler, M., et al., "Biochemical Transfer of Single-Copy Eucaryotic Genes Using Total Cellular DNA as Donor," Cell 14: 725-731 (1978);
H	Wigler, M., et al., "DNA-Mediated Transfer of the Adenine Phosphoribosyltransferase Locus into Mammalian Cells," PNAS 76: 1373-1376 (1979);
I	Willecke, K., et al., "Intraspecies Transfer via Total Cellular DNA of the Gene for Hypoxanthine Phosphoribosyltransferase into Cultured Mouse Cells," Molec. gen. Genet. 170: 179-185 (1979);
J	Graf, L. H., Jr., et al., "Transformation of the Gene for Hypoxanthine Phosphoribosyltransferase," Somatic Cell Genetics 5: 1031-1044 (1979);
K	Pellicer, A., et al., "The Transfer and Stable Integration of the HSV Thymidine Kinase Gene into Mouse Cells," Cell 14: 131-141 (1978);

EXHIBIT	REFERENCE
L	Pellicer, A., et al., "Introduction of a Viral Thymidine Kinase Gene and the Human β-Globin Gene into Developmentally Multipotential Mouse Teratocarcinoma Cells," PNAS 77: 2098-2102 (1980);
M	Wigler, M., et al., "Transformation of Mammalian Cells with an Amplifiable Dominant-Acting Gene," PNAS 77: 3567-3570 (1980);
N	Wigler, M., et al., "Transformation of Mammalian Cells with Prokaryotic and Eukaryotic Genes," Eucaryotic Gene Regulation Proc. ICN-UCLA Symposia, R. Axel and T. Maniatis, editors, Academic Press, pp. 457-475 (1979);
O	Pellicer, A., et al., "Altering Genotype and Phenotype by DNA-Mediated Gene Transfer," Science 209: 1414-1422 (1980); and
P	Perucho, M., et al., "Genetic Physical Linkage of Exogenous Sequences in Transformed Cells," Cell 22: 309-317 (1980).

Respectfully submitted,



John P. White (Reg. No. 28678)
Attorney for Applicants
Tel.: 212-977-9550

UNITED STATES DEPARTMENT OF COMMERCE
Patent and Trademark Office
Address: COMMISSIONER OF PATENTS AND TRADEMARKS
Washington, D.C. 20231

SERIAL NUMBER	FILING DATE	FIRST NAMED APPLICANT	ATTORNEY DOCKET NO.
124,513	02/25/80	RICHARD AXEL	

JOHN P. WHITE
COOPER, DUNHAM, CLARK, GRIFFIN & MORAN
30 ROCKEFELLER PLAZA
NEW YORK, NY 10020

dh

EXAMINER	
E. M. KEPPLINGER	
ART UNIT	PAPER NUMBER
172	4

DATE MAILED: MAILED
July 14 1981
GROUP 170

This is a communication from the examiner in charge of your application.
COMMISSIONER OF PATENTS AND TRADEMARKS

☑ This application has been examined. ☐ Responsive to communication filed on _____ ☐ This action is made final.

A shortened statutory period for response to this action is set to expire 3 month(s), _____ days from the date of this letter.
Failure to respond within the period for response will cause the application to become abandoned. 35 U.S.C. 133

Part I THE FOLLOWING ATTACHMENT(S) ARE PART OF THIS ACTION:

1. ☑ Notice of References Cited by Examiner, PTO-892
2. ☐ Notice of Informal Patent Drawing, PTO-948
3. ☐ Notice of References Cited by Applicant, PTO-1449
4. ☐ Notice of Informal Patent Application, Form PTO-152
5. ☐ _____

Part II SUMMARY OF ACTION

1. ☑ Claims 1-125 are pending in the application.
   Of the above, claims 43, 44, 79, 80, 111, 112, 124 and 125 are withdrawn from consideration.
2. ☐ Claims _____ have been cancelled.
3. ☐ Claims _____ are allowed.
4. ☑ Claims 1-42, 45-78, 81-97, 100-110 and 113-122 are rejected.
5. ☑ Claims 98, 99 and 123 are objected to.
6. ☐ Claims _____ are subject to restriction or election requirement.
7. ☐ The formal drawings filed on _____ are acceptable.
8. ☐ The drawing correction request filed on _____ has been ☐ approved. ☐ disapproved.
9. ☐ Acknowledgment is made of the claim for priority under 35 U.S.C. 119. The certified copy has
   ☐ been received. ☐ not been received. ☐ been filed in parent application, serial no. _____, filed on _____.
10. ☐ Since this application appears to be in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under Ex parte Quayle, 1935 C.D. 11; 453 O.G. 213.
11. ☐ Other

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PTOL-326 (rev. 7-79)

EXAMINER'S ACTION

GENE-CEN 083109

Restriction has been required under 35 USC 121 between the following inventions:

I. Claims 1-42, 45-78, 113, 114, 118 and 119 which are drawn to a process of cotransforming encaryotic cells and the cells produced classified in class 435, subclass 172

II. Claims 43, 44, 79, 80, 111, 112 and 117 which are drawn to proteinaceons material classified in class 260, subclass 112

III. Claims 81-110 which are drawn to a process of cotransforming encaryotic cells with linked DNA classified in class 435, subclass 91

IV. Claims 124 and 125 which are drawn to a process of recovering a gene classified in class 435, subclass 91

V. Claims 115-117 and 120-123 are linking claims which are drawn to a process of producing a biological material, pharmaceutical compositions and method of using them classified in class 424, subclass 177 prosecutable with either Group I or II.

The inventions as grouped are separate and distinct because the processes of Groups I, III and IV do not require the processes of the other Groups. Each of these groups could support a separate patent. Also, the processes of Groups I, III and IV may be used to make products other than those of Group II, such as DNA or vectors for transforming other cells. The products of Group II can be made by other processes such an in genetically transformed procaryotic cells or human beings.

Since these distinct inventions have acquired a separate status in the art, as shown by the above classification, restriction for examination purposes is proper.

Applicants are required to elect a single invention under 35 USC 121 even though this requirement be traversed. In order to be complete, a response must include a proper election.

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In accordance with M.P.E.P. 812.01 a telephone call was made by Examiner Kepplinger, Art unit 172, to applicants' attorney, John P White, on April 29, 1980, who provisionally elected with oral traverse the Group I invention.

To be complete, applicants' response must include an affirmation of the above mentioned provisional election, even through the requirement is traversed.

Claims ~~81-112~~ 43, 44, 79, 80, 111, 112, 124 and 125 stand withdrawn from further consideration by the examiner as being drawn to provisionally non-elected inventions.

EMKEPPLINGER:dh
703-557-3685
6/23/81

Esther M. Kepplinger

ESTHER M. KEPPLINGER
EXAMINER
GROUP ART UNIT 172

GENE-CEN 083111

TO SEPARATE, HOLD TOP AND BOTTOM EDGES —APART AND D... ARBON

FORM PTO-892 (REV. 3-78) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

NOTICE OF REFERENCES CITED 6/12

SERIAL NO.	GROUP ART UNIT	ATTACHMENT TO PAPER NUMBER
124513	172	4

APPLICANT (S): Apel et al

U.S. PATENT DOCUMENTS

		DOCUMENT NO.	DATE	NAME	CLASS	SUB-CLASS	FILING DATE IF APPROPRIATE
	A	3800035	3/74	Moore	435	68	
	B	4195125	3/80	Wacker	435	172	
	C						
	D						
	E						
	F						
	G						
	H						
	I						
	J						
	K						

FOREIGN PATENT DOCUMENTS

		DOCUMENT NO.	DATE	COUNTRY	NAME	CLASS	SUB-CLASS	PERTINENT SHTS. DWG	PERTINENT PP. SPEC
	L	2010847	7/79	Great Britain	Wacker	435	71		
	M								
	N								
	O								
	P								
	Q								

OTHER REFERENCES (Including Author, Title, Date, Pertinent Pages, Etc.)

✓	R	Kretschmer et al, J. Bacteriology 124, 225-231 (1975).
✓	S	Wigler, Cell 11, 223-232 (1977).
	T	Mantei et al, Nature 281, 40-46 (1979).
	U	Lai et al, PNAS 77(1), 244-248 (Jan. 1980).

EXAMINER	DATE	
E. M. Kepplinger	5/22/81	page 1

GENE-CEN 083112

* A copy of this reference is not being furnished with this office action

TO SEPARATE

FORM PTO-892 (REV. 3-78) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

NOTICE OF REFERENCES CITED 6/12

SERIAL NO.	GROUP ART UNIT	ATTACHMENT TO PAPER NUMBER
124513	172	4

APPLICANT (S): Axel et al

U.S. PATENT DOCUMENTS

*		DOCUMENT NO.	DATE	NAME	CLASS	SUB-CLASS	FILING DATE IF APPROPRIATE
	A						
	B						
	C						
	D						
	E						
	F						
	G						
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	I						
	J						
	K						

FOREIGN PATENT DOCUMENTS

*		DOCUMENT NO.	DATE	COUNTRY	NAME	CLASS	SUB CLASS	PERTINENT SHTS. DWG	PERTINENT PP. SPEC.
	L								
	M								
	N								
	O								
	P								
	Q								

OTHER REFERENCES (Including Author, Title, Date, Pertinent Pages, Etc.)

	V 7	Wigler et al, Cell 16, 777-785 (1979). ✓
✓	V 8	Wigler et al, Eucaryotic Gene Regulation, Proc. INRC-UCLA Symposia, RAxel and T. Maniatis, editors, Academic Press, 457-475 (1979).
✓	X 9	Wigler et al, PNAS 76(3), 1373-1376 (1979). ✓
✓	Y 10	Wold et al, PNAS 76(11), 5684-5688 (1979). ✓

EXAMINER	DATE	
E. M. Kepplinger	6/5/81	Page 2

* A copy of this reference is not being furnished with this office action. (See Manual of Patent Examining Procedure, section 707.05 (a).)

GENE-CEN 083113

TO SEPARATE, H... OP AND ...OGES, SNAP—APART AND DISCARD

FORM PTO-892 (REV. 3-78) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

NOTICE OF REFERENCES CITED 6/12

SERIAL NO.	GROUP ART UNIT	ATTACHMENT TO PAPER NUMBER
124513	172	4

APPLICANT (S): Apel et al

U.S. PATENT DOCUMENTS

*		DOCUMENT NO.	DATE	NAME	CLASS	SUB-CLASS	FILING DATE IF APPROPRIATE
	A						
	B						
	C						
	D						
	E						
	F						
	G						
	H						
	I						
	J						
	K						

FOREIGN PATENT DOCUMENTS

*		DOCUMENT NO.	DATE	COUNTRY	NAME	CLASS	SUB-CLASS	PERTINENT SHTS DWG	PERTINENT PP. SPEC.
	L								
	M								
	N								
	O								
	P								
	Q								

OTHER REFERENCES (Including Author, Title, Date, Pertinent Pages, Etc.)

✓	RR	The Merck Index, 8th Edition, Merck + Co., Inc., Rahway, N.J., 568 (1968). ✓
/	SS	Szybalska et al PNAS 48, 2026-2034 (1962). —
/	TT	McCutchan et al, Journal National Cancer Institute, 41, 351-358 (1963) —
/	UU	Wigler et al, Cell 14, 725-731 (1978). —

EXAMINER	DATE	
E. M. Kipplinger	6/5/81	page 3

GENE-CEN 083114

* A copy of this reference is not being furnished with this office action. (See Manual of Patent Examining Procedure, section 707.05 (a).)

TO SEPARATE, HOLD TOP AND BOTTOM EDGES, SNAP—APART AND DISCARD C ON

FORM PTO-892 (REV. 3-78) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

NOTICE OF REFERENCES CITED 6/12

SERIAL NO.	GROUP ART UNIT	ATTACHMENT TO PAPER NUMBER
124513	172	4

APPLICANT (S): Axel et al

U.S. PATENT DOCUMENTS

*		DOCUMENT NO.	DATE	NAME	CLASS	SUB-CLASS	FILING DATE IF APPROPRIATE
	A						
	B						
	C						
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	J						
	K						

FOREIGN PATENT DOCUMENTS

*		DOCUMENT NO.	DATE	COUNTRY	NAME	CLASS	SUB-CLASS	PERTINENT SHTS. DWG	PERTINENT PP. SPEC
	L								
	M								
	N								
	O								
	P								
	Q								

OTHER REFERENCES (Including Author, Title, Date, Pertinent Pages, Etc.)

✓	V	Willecke et al, Molec. gen. Genet. 170, 179-185 (1979).
✓	W	Graf et al, Somatic Cell Genetics 5, 1031-1044 (1979).
✓	X	Pellicer et al, Cell 14, 131-141 (1978).
✓	Y	Mercola et al, Science 208, 1033-1035 (May 1980).

EXAMINER	DATE	
E. M. Kepplinger	6/5/81	page 4

* A copy of this reference is not being furnished with this office action. (See Manual of Patent Examining Procedure, section 707.05 (a).)

GENE-CEN 083115

17668-A JPW

#5

9/15/81 R.B.

SEP 11 10 51 AM '81

GROUP 170

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Richard Axel, et al.

Serial No.: 124,513 Examiner: Esther Kepplinger

Filed : February 25, 1980 Group Art Unit: 172

For : PROCESSES FOR INSERTING DNA INTO EUCARYOTIC CELLS AND FOR PRODUCING PROTEINACEOUS MATERIALS

30 Rockefeller Plaza
New York, New York 10112

10 September 1981

Honorable Commissioner of Patents
and Trademarks
Washington, D.C. 20231

Sir:

FIRST REQUEST FOR A ONE MONTH EXTENSION OF TIME

It is respectfully requested that the period for filing a response to the July 14, 1981 Office Action, now set to expire October 14, 1981, be extended one month to expire November 14, 1981.

This is the first request for an extension of time for filing a response to the outstanding Office Action and it is not anticipated that another request will be submitted.

After the Office Action dated July 14, 1981 was received, a letter reporting on this Office Action, together with copies of the Office Action and of the Examiner was promptly mailed to applicants. Nevertheless, in view of the numerous grounds for rejection set forth in the Action and the large number of cited references, Applicants and Applicants' undersigned attorney require additional time to confer and to prepare a complete response to the Office Action.

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GENE-CEN 083116

It is therefore requested that a one month extension of the deadline for filing a response, from October 14, 1981 to November 14, 1981, be favorably considered and granted.

Respectfully submitted,

John P White

John P. White
Reg. No. 28,678
Attorney for Applicant
(212) 977-9550

Approved
James
9/11/81

D17668-A JPW

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Richard Axel, et al.

Serial No. : 124,513 Examiner: Esther M. Kepplinger

Filed : February 25, 1980 Group Art Unit: 172

For : PROCESSES FOR INSERTING DNA INTO EUCARYOTIC CELLS AND FOR PRODUCING PROTEINACEOUS MATERIALS

30 Rockefeller Plaza
New York, New York 10112

5 November 1981

Honorable Commissioner of Patents
and Trademarks
Washington, D.C. 20231

Sir:

AMENDMENT

This Amendment is submitted in response to the July 14, 1981 Office Action issued in connection with the above-identified application. A response to that Office Action is presently due no later than November 14, 1981. Please amend the subject application as follows:

IN THE CLAIMS

In Claim 32, change "30" to --31--.

In Claim 113, between "for a" and "phenotype not expressed", insert --selectable--.

Cancel Claims 10, 11, 22, 55, 56, 91, 92, 117 and 120-123.

Amend Claims 1 and 23 as follows:

--1. (amended) A process for inserting foreign DNA I into a eucaryotic cell which comprises cotransforming said eucaryotic cell with said foreign DNA I and with unlinked foreign DNA II which codes for a selectable phenotype not expressed by said eucaryotic cell, said cotransformation being carried out in a suitable medium and in the presence of selective conditions

GENE-CEN 083118

permitting survival or identification of eucaryotic cells which have acquired said selectable phenotype[.], said foreign DNA I being incorporated into the chromosomal DNA of said eucaryotic cell.

--23. (amended) A process for inserting purified foreign DNA I coding for proteinaceous material which is not associated with a selectable phenotype into a eucaryotic cell which comprises co-transforming said eucaryotic cell with said foreign DNA I coding for proteinaceous material which is not associated with a selectable phenotype and with physically unlinked foreign DNA II coding for proteinaceous material which is associated with a selectable phenotype, said cotransformation being carried out in the presence of selective conditions permitting survival or identification of eucaryotic cells which have acquired said selectable phenotype[.], said foreign DNA I being incorporated into the chromosomal DNA of said eucaryotic cell.--

REMARKS

In the July 14, 1981 Office Action, the Examiner reiterated the restriction requirement previously entered in the subject application. As indicated in the Office Action, Applicants' undersigned attorney provisionally elected Invention I, Claims 1-42, 45-78, 113, 114, 118 and 119 with traverse on April 29, 1980. However, the Examiner carried out an extended International Search in a corresponding International Application under the Patent Cooperation Treaty. As a result, the July 14, 1981 Office Action includes an examination on the merits with respect to Claims 1-42, 45-78, 81-99, 100-110 and 113-123. In the Office Action, Claims 43, 44, 79, 80, 111, 112, 124 and 125 were held withdrawn from consideration at this time. This communication responds to all of the grounds for rejection and objection set forth in the July 14, 1981 Office Action wherein Claims 1-42, 45-78, 81-97, 100-110 and 113-122 were rejected and Claims 98, 99 and 123 were objected to.

In view of the extensive number of rejections asserted by the Examiner, Applicants will discuss the rejections using the following format. The grounds for each rejection or objection will be separately reiterated, followed by the reasons urged by Applicants for withdrawing the rejection or objection. A request for reconsideration is not separately stated with respect to each ground for rejection or objection, but is made now and again in the Summary at the end of this Amendment with respect to all of the grounds asserted by the Examiner.

As a result of this Amendment, the claims now under examination in this application are the following: 1(as amended), 2-9, 12-21, 23(as amended), 24-31, 32(as amended), 33-42, 45-54, 57-78, 81-90, 93-110, 113(as amended), 114-116 and 118-119.

I. REJECTIONS UNDER 35 U.S.C. § 112

1. Claim 32 was rejected under 35 U.S.C. § 112, 2nd paragraph, on the ground that the claim is allegedly indefinite. The Examiner suggested that dependence from Claim 30 was improper and suggested that Claim 31 was intended.

Applicants agree with the Examiner and have amended Claim 32 as suggested.

2. Claim 113 was rejected under 35 U.S.C. § 112, 1st paragraph, on the ground that the claim is allegedly based on an insufficient disclosure in failing to define the phenotype as "selectable". According to the Examiner, "The transformed cells can be identified only if they can be selected. Thus, it is essential."

Applicants disagree with the Examiner's stated reason for rejecting Claim 113, namely, that the claim is based upon an insufficient disclosure. Nevertheless, Applicants agree that Claim 113 should define the phenotype as "selectable" and have so amended Claim 113.

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3. Claims 3-8, 24, 30, 32, 36, 40, 47-52, 69, 72, 104, 108, 76, 116 and 83-88 were rejected under 35 U.S.C. § 112, 1st paragraph, as allegedly based on a specification which is non-enabling in regard to the various proteins claimed. According to the Examiner, "The specification does not show insertion of any of the proteins claimed. It is not seen that one skilled in the art could practice the invention."

Applicants are somewhat confused as to the precise basis for the Examiner's rejection. Nevertheless, Applicants will attempt to respond by making certain assumptions regarding the basis for the rejection and then replying thereto.

If the Examiner's position is based upon an alleged lack of disclosure of the specific proteins claimed, Applicants direct the Examiner to various portions of the Specification, for example, page 12, lines 3-7, where full support may be found for the claims.

If the Examiner's position is based upon the fact that none of the proteins claimed is described in the "Experimental Details" portion of the application, Applicants respond that 35 U.S.C. § 112 does not require that the process invented and claimed by Applicants be used to make specific claimed products. 35 U.S.C. § 112, 1st paragraph, only requires that, "The Specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor of carrying out his invention." Thus, there is no requirement that Applicants show actual reduction to practice of their process for specific, claimed proteins.

If the Examiner's position is that the application does not contain a written description of the invention sufficient to enable one skilled in the art to use the invention, Applicants reply that the application does contain a sufficient written

description. In support of their position, Applicants direct the Examiner's attention to the Detailed Description of the Invention, pages 9-86 of the application. In addition, Applicants direct the Examiner's attention to several cited references, each of which establishes that persons skilled in the art, upon reading disclosures of the invention identical to the disclosure set forth in the application were able to use the invention.

Thus, cited reference R, Mantei, N., et al., Nature, Vol. 281, pp.40-46 (1979) refers to and shows use of Applicants' cotransformation system. It also refers to published papers coauthored by Applicants. Specifically, the Mantei, et al. article includes, as footnoted reference 41, Wigler, M., et al., Cell, Vol. 16, pp.777-785 (1979). This Wigler, et al. article, cited reference V of the Office Action, is referred to on page 22, line 20, of the application where Applicants indicate that the "First Series of Experiments" set forth at pages 22-35 of the application are also set forth in this publication.

Similarly, Lai, Eugene C., et al., PNAS, Vol 77, pp.244-248 (1980), cited reference U, describes "the transformation of mouse LMTK$^-$ cells with the chromosomal ovalbumin gene by using this DNA-mediated gene transfer system." The system referred to is identified in footnoted references 8 and 9, the aforementioned Mantei, et al. and Wigler, et al. articles.

Likewise, cited reference YY, Mercola, K.M., et al., Science, Vol. 208, pp.1033-1036 (1980) describes insertion of a gene into the bone marrow of mice, indicating on page 1033 that, "We used the technique of Wigler, et al.[1]."

These cited references clearly establish that Applicants' description of the invention, as identically set forth in both this application and in various publications, is sufficient to enable one skilled in the art to use the invention. In fact, as these references show, others skilled in the art have used these descriptions as a basis for practicing the invention and for further experimentation.

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In view of the foregoing, Applicants maintain that the application describes their invention in sufficient detail to enable one skilled in the art to use the invention.

4. Claims 11, 56 and 92 were rejected under 35 U.S.C. § 112, 1st paragraph, on the ground that the claims allegedly are based on a specification which is non-enabling in regard to having the DNA encapsidated in a phage. According to the Examiner, "It is not shown and one skilled in the art would not know how to practice the invention. No best mode is shown."

Although Applicants disagree with the Examiner's stated basis for rejection, Applicants are cancelling Claims 11, 56 and 92 at this time in order to advance prosecution of their application. However, cancellation of these claims is made without prejudice to Applicants' right to present the claims in a subsequent application derived from this application.

5. Claims 31-42, 71-78, 103-110, 115-117 and 119-123 were rejected under 35 U.S.C. § 112, 1st paragraph, on the ground that the claims allegedly are based on a specification which is non-enabling in regard to producing protein products. The Examiner stated, "Applicants do not show any production and recovery (although the globin gene is inserted, no protein is recovered); no best mode is shown. One skilled in the art could not practice the invention."

Initially, Applicants point out that the Examiner errs regarding Applicants' alleged failure to shown production and recovery of protein. Although Applicants do not disclose recovery of globin protein, production of rabbit β-globin would be clear to one skilled in the art given the fact that expression of the rabbit β-globin gene to form RNA was shown by hybridization experiments. See pages 48-49 of the application.

More importantly, production and recovery of protein is described on page 65 and in Table 1 on page 66. As set forth, production of the protein, thymidine kinase, was verified by assaying the ability of HSV-tk-specific antibody to neutralize enzymatic activity in extracts of transformed clones. Production and recovery was further shown by electrophoretic analysis.

Applicants also assert that their application sets forth the best mode contemplated by them for carrying out the invention and describes the invention in sufficient detail to enable one skilled in the art to practice the invention and to produce and recover protein products.

Two of the references cited by the Examiner, reference U [Lai, E.C., et al., PNAS, Vol. 77, pp.244-248 (1980)] and reference WW [Graff, L.H., et al., Somatic Cell Genetics, Vol. 5, pp.1031-1044 (1979)], illustrate the fact that others skilled in the art were able to produce and recover protein using Applicants' invention. Thus, in reference U, the authors report that they used the DNA-mediated gene transfer system of Wigler, et al. [Cell, Vol. 16, pp.777-785 (1979)] to transform mouse cells with an ovalbumin gene and detected the ovalbumin polypeptide in the transformants. Similarly, in reference WW, the authors report that they used the transformation system of Wigler, et al. to produce the protein hypoxanthine phosphoribosyltransferase as confirmed by enzymatic activity in extracts of transformed cells.

In view of the foregoing, Applicants maintain that their application sufficiently teaches the production and recovery of protein to satisfy the requirements of 35 U.S.C. § 112.

6. Claims 10, 11, 55, 56, 91 and 92 were rejected under 35 U.S.C. § 112, 2nd paragraph, on the ground that the claims allegedly contradict independent claims which recite unlinked DNA. The Examiner stated, "The alternative and DNA I and DNA II being attached to other DNA contradicts that."

Although Applicants disagree with the Examiner's stated basis for rejection, Applicants are cancelling Claims 10, 11, 55, 56, 91 and 92 at this time in order to advance prosecution of this application. However, cancellation of these claims is made without prejudice to Applicants' right to present the claims in a subsequent application derived from this application.

II. REJECTIONS UNDER 35 U.S.C. § 102 and/or § 103

1. Claims 1-42 were rejected under 35 U.S.C. § 103 on the basis that the claimed invention would allegedly have been obvious to one skilled in the art at the time made given the teachings of reference R, Kretschmer, et al., J. Bacteriology, Vol. 124, pp.225-231 (1975), in view of the disclosure of reference S, Wigler, et al., Cell, Vol. 11, pp.223-232 (1977). The Examiner stated, "R shows cotransformation of procaryotic cells with unlinked pieces of DNA, one of which has selectable characteristics. S shows eucaryotic cells capable of transformation with gene coding for selectable characteristic. Combination suggests eucaryotes capable of cotransformation with unlinked DNA also since single gene transformation is successful. Use of gene for any protein would be obvious. Phage vectors and calcium phosphate treatment are conventional and obvious."

As set forth more fully on pages 1-5 of the application, Applicants maintain that their invention is not obvious over the teachings of Kretschmer, et al. (reference R) either alone or in combination with other teachings for at least the following reasons:

a. Applicants' invention is in a different field from the prior teachings concerning the modification of procaryotic cells, e.g. bacteria. Applicants' invention involves eucaryotic cells which have long been recognized by those skilled in the art as different from procaryotic cells for numerous reasons, including those set forth at lines 15-23 on page 1.

b. Applicants' invention provides major advantages over bacterial systems as indicated at line 35, page 3 to line 14, page 5.

c. Applicants' invention involves integration of foreign DNA in the chromosomal DNA of the cotransformed eucaryotic cell. The Kretschmer, et al. publication involves plasmids, extrachromosomal DNA molecules, which are not chromosomally integrated in cotransformed procaryotic cells. As a result, the bacterial cotransformants lose acquired

traits, whereas the eucaryotic cotransformants can be stably reproduced. Applicants have amended Claims 1 and 23 to more clearly define their invention and distinguish over work with bacterial systems.

In view of the foregoing, Applicants maintain that one skilled in the art of eucaryotic cell manipulations would not consider it obvious that processes applicable to bacterial cells would be applicable to eucaryotic cells. Moreover, based upon the bacterial work, one would not expect integration of foreign DNA into the chromosomes of eucaryotic cells, but rather extra-chromosomal replication of foreign DNA. In this respect, the Kretschmer, et al. article teaches away from Applicants' invention. Accordingly, Applicants maintain that there is no teaching or suggestion in Kretschmer, et al. which renders their invention obvious.

As for the combination of the teachings of Kretschmer, et al. with those of Wigler, et al., Cell, Vol. 11, pp.223-232 (1977), Applicants maintain that such a combination involves the use of non-analogous prior art. Moreover, the cited Wigler, et al. article neither teaches or suggests cotransformation in eucaryotic cells. Therefore, combining a teaching of cotransformation in bacterial cells with a teaching of transformation in eucaryotic cells is not proper, and, more importantly, does not result in Applicants' claimed invention.

2. Claims 28-30, 67 and 70 were rejected under 35 U.S.C. § 102 on the grounds that the claims are allegedly fully met by either reference B, Wacker, U.S. Patent No. 4,195,125 (1980), or reference L, corresponding British Patent 2,010,847 (1979).

The disclosures of these references are substantially identical and relate to processes for fusing cells and to the hybrid fusion products which result. Applicants' rejected claims

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are not directed to hybrid fusion products created by cell fusion. Accordingly, Applicants maintain that the Examiner's rejection under 35 U.S.C. § 102 is erroneous since the cited references neither teach, suggest, nor relate to Applicants' claimed invention.

3. Claims 45-64 and 71-78 were rejected under 35 U.S.C. § 103 on the ground that the claimed invention allegedly would have been obvious at the time made to one skilled in the art given the teachings of Kretschmer, et al. (reference R) and Mantei, et al. (reference T). According to the Examiner, "R shows cotransformation of procaryotes with unlinked DNA one of which codes for a selectable characteristic. T shows transforming L cells with linked globin and TK DNA by mixing high ratios of globin DNA and TK DNA to introduce multiple copies of the β-globin. Combination suggests transformation of eucaryotes with unlinked DNA in amounts of high protein DNA to indicator DNA will insert multiple copies. Use of DNA for other proteins obvious. Phage vectors are conventional and obvious."

Applicants' position, namely, that the Kretschmer, et al. article, neither singly nor in combination with other references, teaches or suggests their invention is set forth in Section II, point 1, hereinabove, and the Examiners' attention is directed to the argument set forth there. In addition, the argument there advanced in opposition to an obviousness rejection based upon combining the teachings of Kretschmer, et al. with those of the Wigler, et al. (reference S) are also applicable to this rejection, particularly since the Mantei, et al. article involves linked DNA which is distinguishable from Applicants' unlinked DNA. Combining the teachings of Kretschmer, et al. with those of Mantei, et al. is not proper and, more importantly, does not result in Applicants' claimed invention.

Finally, the Mantei, et al. paper was published on September 6, 1979, less than one year prior to the filing date of this application. As set forth more fully in the accompanying DECLARATION UNDER 37 C.F.R. § 1.131, Applicants' invention was conceived at least as early as February 25, 1979 and reduced to practice in the United States at least as early as February 25, 1980, either actually with respect to certain embodiments or

constructively with respect to others. With respect to all embodiments, Applicants proceeded diligently from the date of conception to a reduction to practice of the invention. Accordingly, Applicants request that the Mantei, et al. article be removed as a reference and contend that once the reference is removed, the present ground for rejection is obviated.

4. Claims 25-29, 65-68, 70, 101 and 102 were rejected under 35 U.S.C. § 102 or § 103 on the ground that the invention claimed allegedly is anticipated by or would have been obvious when made to one skilled ian the art given the teachings of Lai, et al. (reference U) or Mantei, et al. (reference T). According to the Examiner, "Both show eucaryotic cells transformed by linked protein DNA and TK DNA. No difference in the transformed cell is seen whether linked or unlinked DNA is used."

Applicants initially wish to point out that the Examiner is in error regarding the Lai, et al. reference. This reference involves unlinked cotransformation, not linked as urged by the Examiner. This article was published in January 1980, less than one year prior to the filing date of this application.

With respect to the Mantei, et al. reference, Applicants reiterate their position that use of linked DNA is patentably distinguishable from their claimed invention.

Applicants again direct the Examiner's attention to the accompanying DECLARATION UNDER 37 C.F.R. § 1.131 and request that the Lai, et al. and the Mantei, et al. references be removed. Once removed, Applicants maintain that the rejection is obviated.

5. Claims 30, 81-97, 100 and 103-110 were rejected under 35 U.S.C. § 103 on the ground that the claimed invention allegedly would have been obvious at the time made to one skilled in the art given the teachings of Lai, et al. (reference U) or Mantei, et al. (reference T). According to the Examiner, "U shows transforming L cells with TK gene linked to ovalbumin gene so that multiple copies of the ovalbumin gene are inserted and ovalbumin is synthesized. T shows transforming L cells with β-globin DNA and TK DNA linked so multiple copies of globin gene are inserted into cell. Obvious to use other protein genes and eucaryotic cells."

Applicants' argument set forth in Section II, point 4, is equally pertinent to this rejection. Applicants therefore direct the Examiner's attention to this argument and to the

accompanying DECLARATION UNDER 37 C.F.R. § 1.131. In view of the latter, Applicants again request that the Examiner remove the Lai, et al. and Mantei, et al. references. Once removed, Applicants maintain that the subject rejection is obviated.

6. Claims 1,2,9, 10, 12, 14, 16, 17-23, 25-29, 45, 46, 53-55, 58, 60-68, 70, 113, 114 and 118 were rejected under 35 U.S.C. § 102 on the ground that the claims allegedly are fully met by Wigler, et al., Eucaryotic Gene Regulation, Proc. ICN-UCLA Symposium, R. Axel and T. Maniatis, editors, Academic Press, pp.457-475 (1979) (reference W), or Wigler, et al., Cell, Vol. 16, pp.777-785 (1979) (reference V) or Wigler, et al., PNAS, Vol. 76, pp.1373-1376 (1979) (reference X).

Each of references W, V and X is coauthored by Applicants.

The disclosure of reference W is substantially identical to the "Third Series of Experiments" set forth on pages 45-53 of the application. Reference W was published in December 1979, less than one year prior to the filing date of the subject application. In support of the fact that reference W was published in December 1979, Applicants submit copies of correspondence between Applicants' attorney and the publisher, Academic Press. (Exhibit A).

The disclosure of reference V is substantially identical to the "First Series of Experiments" set forth on pages 22-35. Reference V was published in April 1979, less than one year prior to the filing date of the subject application.

The disclosure of reference X does not relate to cotransformation. Accordingly, the rejection under 35 U.S.C. § 102 is improper. In addition, reference X was published in March 1979, less than one year prior to the filing date of the subject application.

Applicants again direct the Examiner's attention to the accompanying DECLARATION UNDER 37 C.F.R. § 1.131 and request that the Examiner remove references W, V and X, each of which was coauthored by Applicants and published less than one year prior to the filing date of the subject application.

7. Claims 3-8, 11, 13, 15, 24, 30-42, 47-52, 56, 57, 59, 69, 71-78, 115-117 and 119 were rejected under 35 U.S.C. § 103 on the ground that the claimed invention allegedly would have been obvious when made to one skilled in the art given the teachings of either reference W, reference V or reference X. According to the Examiner, "All three show cotransformation of eucaryotic cells with unlinked DNA of which one codes for a selectable characteristic. Use of DNA for various proteins obvious. Recovery of protein and transformation of other eucaryotic cells would be obvious.

Applicants direct the Examiner's attention to their remarks under immediately preceding point 6. In addition, Applicants reiterate their request that references W, V and X be removed in view of the accompany DECLARATION UNDER 37 C.F.R. § 1.131.

8. Claims 1, 2, 9, 10, 12, 14, 16-18, 22, 23, 25-29, 45, 46, 53-55, 58, 60, 61, 64, 65-68, 70, 113, 114 and 118 were rejected under 35 U.S.C. § 102 on the ground that the claims allegedly are fully met by Wold, et al., PNAS, Vol. 76, pp.5684-5688 (1979) (reference Y).

Reference Y is also coauthored by Applicants. Its disclosure is substantially identical to the "Second Series of Experiments" set forth on pages 36-45. Reference Y was published in November 1979, less than one year prior to the filing date of the subject application.

Once again, Applicants direct the Examiner's attention to the accompanying Rule 131 Declaration and request that the Examiner also remove reference Y.

9. Claims 3-8, 11, 13, 15, 19-21, 24, 30-42, 47-52, 56, 57, 59, 62, 63, 69, 71-78, 115-117 and 119 were rejected under 35 U.S.C. § 103 on the ground that the claimed invention allegedly would have been obvious at the time made to one skilled in the art given the teachings of reference Y. According to the Examiner, "Y shows cotransformation of eucaryotic cells with unlinked DNA of which one codes for a selectable characteristic. Use of DNA for various proteins, recovery of protein and transformation of other eucaryotic cells would be obvious.

Applicants direct the Examiner's attention to their remarks under immediately preceding point 8. In addition, Applicants reiterate their request that the reference Y be removed in view of the accompanying DECLARATION UNDER 37 C.F.R. § 1.131.

10. Claim 120 was rejected under 35 U.S.C. § 102 on the ground that the claim is fully met by The Merck Index, 8th Edition, Merck & Co., Inc., Rahway, New Jersey, 07568 (1968) (reference RR).

Although Applicants disagree with the Examiner's stated basis for rejection, Applicants are cancelling Claim 120 at this time in order to advance prosecution of their application. However, cancellation of Claim 120 is made without prejudice to Applicants' right to again present the claim in a subsequent application derived from this application.

11. Claims 117, 121 and 122 were rejected under 35 U.S.C. § 102 on the ground that these claims allegedly are fully met by Goore, U.S. Patent 3,800,035 (1974).

Once again Applicants, although disagreeing with the Examiner's stated basis for rejection, are cancelling Claims 117, 121 and 122 at this time in order to advance prosecution of their application. Cancellation of these claims is made without prejudice to Applicants' right to present the claims in a subsequent application derived from this application.

III. OBJECTIONS

1. The Specification was objected to under 35 U.S.C. § 112, 1st paragraph, as allegedly nonenabling because it does not set forth enough information for one skilled in the art to practice the invention as claimed; does not show inserting or producing the various proteins claimed; and does not show a best mode. Additionally, the Examiner stated that, in regard to products which are partially protein, the Specification only speculates that it should be possible which allegedly is not an enabling disclosure. Finally, the Examiner stated that one skilled in the art could not practice the invention.

Applicants understand the reasons asserted for objecting to the specification to be identical to those asserted by the Examiner in rejecting the claims. Accordingly, Applicants do not reiterate their previously set forth position that the application contains a sufficient disclosure of the invention to enable one skilled in the art to practice the invention and that the application sets forth the best mode contemplated by Applicants.

With respect to partially proteinaceous products, Applicants maintain that the application is enabling since it teaches one skilled in the art how to practice the invention. An actual experiment involving partially proteinaceous products is not required under 35 U.S.C. § 112.

2. Claim 123 was objected to as an improper multiply dependent claim since it allegedly is not in proper form as required by 37 C.F.R. § 1.75 and therefore was not treated on its merits. In this regard, the Examiner directed Applicants' attention to M.P.E.P. § 608.01(n).

In order to advance prosecution of the application, Claim 123 is being cancelled at this time without prejudice to Applicants' right to present the claim in a subsequent application derived from this application.

3. Claims 98 and 99 were objected to on the cover sheet of the July 14, 1981 Office Action. No further reference to these claims or to the basis for the objection was set forth.

Applicants have reviewed Claims 98 and 99 but find no basis for an objection to these claims. Applicants therefore request that the Examiner reconsider any objection to them.

IV. SUMMARY

In summary, Applicants maintain that the claims now pending in this application accurately define their invention, and that the claims are patentably distinguishable over the prior art, particularly in view of the accompanying DECLARATION UNDER 37 C.F.R. § 1.131. Accordingly, Applicants request that the Examiner reconsider the various grounds of rejection and objection set forth in the outstanding Office Action and earnestly solicit allowance of the claims now pending.

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If a telephone interview would assist in advancing prosecution of this application, Applicants' undersigned attorney invites the Examiner to telephone him at the number provided.

No fee is deemed necessary in connection with this Amendment. If a fee is required, authorization is hereby given to charge the amount of any such fee to Deposit Account No. 03-3125.

Respectfully submitted,

John P White

John P. White
Reg. No. 28,678
Attorney for Applicant
212-977-9550

I hereby certify that this paper is being deposited this date with the U.S. Postal Service as first class mail addressed to Commissioner of Patents and Trademarks, Washington, D. C. 20231.

John P White 11/5/81
John P. White Date
Reg. No. 28,678

D17668-A JPW

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Richard Axel, et al.

Serial No.: 124,513 Examiner: Esther M. Kepplinger

Filed : February 25, 1980 Group Art Unit: 172

For : PROCESSES FOR INSERTING DNA INTO EUCARYOTIC CELLS AND FOR PRODUCING PROTEINACEOUS MATERIALS

30 Rockefeller Plaza
New York, New York 10112

5 November 1981

Honorable Commissioner of Patents
and Trademarks
Washington, D.C. 20231

Sir:

COMMUNICATION

Applicants herewith submit a Declaration Under 37 C.F.R. § 1.131 signed by Richard Axel and Saul J. Silverstein and an identical Declaration signed by Michael H. Wigler. This Declaration is submitted along with an Amendment dated 5 November 1981.

Respectfully submitted,

I hereby certify that this paper is being deposited this date with the U.S. Postal Service as first class mail addressed to Commissioner of Patents and Trademarks, Washington, D. C. 20231.
John P. White 11/5/81
John P. White Date
Reg. No. 28,678

John P. White
Reg. No. 28,678
Attorney for Applicants
212-977-9550

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D17668-A JPW

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Richard Axel, et al.

Serial No. : 124,513 Examiner: Esther M. Kepplinger

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30 Rockefeller Plaza
New York, New York 10112

Honorable Commissioner of Patents
and Trademarks
Washington, D.C. 20231

Sir:

DECLARATION UNDER 37 C.F.R. § 1.131

We, RICHARD AXEL, MICHAEL H. WIGLER and SAUL J. SILVERSTEIN, declare as follows:

1. We are the RICHARD AXEL, MICHAEL H. WIGLER and SAUL J. SILVERSTEIN who are named as Applicants in the above-identified patent application.

noted
Ewk
1/20/82

2. We have read the Office Action issued in connection with the subject application on July 14, 1981. We have also read the references cited therein, including the following:

T: Mantei, N., et al., Nature, Vol. 281, pp.40-46, published September 6, 1979;

U: Lai, E.C., et al., PNAS, Vol. 77, pp.244-248, published January 1980;

V: Wigler, M., et al., Cell, Vol. 16, pp.777-785, published April 1979;

W: Wigler, M., et al., Eucaryotic Gene Regulation, Proc. ICN-UCLA Symposium, R. Axel and T. Maniatis, editors, Academic Press, pp.457-475, published December 1979;

X: Wigler, M., et al., PNAS, Vol. 76, pp.1373-1376, published March 1979;

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Y: Wold, B., et al., PNAS, Vol. 76, pp.5684-5688, published November 1979.

3. The invention described and claimed in the subject application was conceived by us at least as early as February 25, 1979.

4. Subsequent to our conception, we, either personally or through persons working under our direct supervision, conducted experiments in the United States reducing the invention to practice. The results of these experiments are set forth in the subject application and in various articles coauthored by us.

5. Specifically, one series of experiments involving the cotransformation process is set forth as the "First Series of Experiments" on pages 22-35 of the application. These experiments are also described in coauthored reference V, Wigler, M., et al., Cell, Vol. 16, pp.777-785, published April 1979. As this publication indicates, the manuscript was received by Cell on January 8, 1979. Therefore, it is clear that an actual reduction to practice of this embodiment of our invention was completed prior to January 8, 1979.

6. Another series of experiments involving cotransformation with multiple DNA molecules is set forth as the "Second Series of Experiments" on pages 36-45 of the application. These experiments are also described in coauthored reference Y, Wold, et al., PNAS, Vol. 76, pp.5684-5688, published November 1979. As this publication indicates, the manuscript was communicated to PNAS on July 16, 1979. Therefore, it is clear that an actual reduction to practice of this embodiment of our invention was completed prior to July 16, 1979.

7. Another series of experiments involving cotransformation is set forth as the "Third Series of Experiments" on pages 45-53 of the application. These experiments are also described in coauthored reference W, Wigler, M., et al., Eucaryotic Gene Regulation, Proc. ICN-UCLA Symposium, R. Axel and T. Maniatis, editors, Academic Press, pp.457-475, published December 1979. It is clear that an actual reduction to practice of this embodiment of our invention was completed prior to December 1979.

8. Another series of experiments involving amplification of cotransformed genes is set forth as the "Fourth Series of Experiments" on pages 54-62 of the application. These experiments were not published prior to the February 25, 1980 filing date of this application, but were subsequently published as the coauthored article, Wigler, M., et al., PNAS, Vol. 77, pp.3567-3570, in June 1980. This manuscript was communicated to PNAS on March 10, 1980. The experiments described were carried out at least as early as February 25, 1980.

9. A final series of experiments involving production of protein by cotransformed genes is set forth as the "Fifth Series of Experiments" on pages 63-74 of the application. These experiments were not published prior to the Feburary 25, 1980 filing date of this application, but were subsequently published as the coauthored article, Pellicer, A., et al., PNAS, Vol. 77, pp.2098-2102, published April 1980. This manuscript was communicated to PNAS on January 10, 1980. The experiments described were completed at least as early as December 31, 1979.

10. Another series of experiments involving transformation was carried out by us. These experiments are not described in the application and do not relate to unlinked cotransformation.

These experiments are described in coauthored reference X, Wigler, M., et al., PNAS, Vol. 76, pp.1373-1376, published March 1979. As this publication indicates, the manuscript was communicated to PNAS on December 14, 1978. Therefore, it is clear that the experiments described were completed prior to December 14, 1978.

11. As stated in preceding paragraphs 5, 6, 7 and 10, cited references V, Y, W and X, respectively, were coauthored by us. All were published less than one year prior to the February 25, 1980 filing date of this application. References V, Y and W describe embodiments of the invention conceived and actually reduced to practice in the United States prior to the publication dates of these references. Reference X describes experiments actually performed in the United States prior to the publication date of this reference.

12. Reference T, Mantei, N., et al., Nature, Vol. 281, pp.40-46 was published on September 6, 1979, less than one year prior to the February 25, 1980 filing date of this application. The Mantei, et al. paper concerns transformation with linked genes, not unlinked cotransformation. Moreover, the Mantei, et al. article does not relate to amplification of transformed genes. In addition, as clearly indicated by the second to the last paragraph of the Mantei, et al. paper, Applicants "reported cotransformation of mouse L cells with rabbit β-globin DNA and HSVI-TK DNA" in Wigler, M., et al., Cell, Vol. 16, pp.777-785, published April 1979, prior to this Mantei, et al. publication. As stated in paragraph (6) hereinabove, we actually reduced to practice the embodiment of our invention relating to cotransformation with multiple copies of transformed genes at least as early as July 16, 1979.

13. Reference U, Lai, E.C., et al., PNAS, Vol. 77, pp.244-248 was published in January 1980, less than one year prior to the February 25, 1980 filing date of this application. The Lai, et al. paper concerns use of Applicants' cotransformation process to introduce into eucaryotic cells the gene for ovalbumin and produce polypeptide. See last eight lines of the first full paragraph (preceding Materials and Methods). As stated in paragraph (9) hereinabove, we demonstrated production of protein by cotransformation at least as early as December 31, 1979.

We declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that willful false statements may jeopardize the validity of the application or any patent issuing thereon.

_____	_____
DATE	RICHARD AXEL
Nov 1, 1981	Michael H Wigler
DATE	MICHAEL H. WIGLER
_____	_____
DATE	SAUL J. SILVERSTEIN

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D17668-A JPW

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants : Richard Axel, et al.

Serial No. : 224,513 Examiner: Esther M. Kepplinger

Filed : February 25, 1980 Group Art Unit: 172

For : PROCESSES FOR INSERTING DNA INTO EUCARYOTIC CELLS AND FOR PRODUCING PROTEINACEOUS MATERIALS

30 Rockefeller Plaza
New York, New York 10112

Honorable Commissioner of Patents
and Trademarks
Washington, D.C. 20231

Sir:

DECLARATION UNDER 37 C.F.R. § 1.131

We, RICHARD AXEL, MICHAEL H. WIGLER and SAUL J. SILVERSTEIN, declare as follows:

1. We are the RICHARD AXEL, MICHAEL H. WIGLER and SAUL J. SILVERSTEIN who are named as Applicants in the above-identified patent application.

2. We have read the Office Action issued in connection with the subject application on July 14, 1981. We have also read the references cited therein, including the following:

T: Mantei, N., et al., Nature, Vol. 281, pp.40-46, published September 6, 1979;

U: Lai, E.C., et al., PNAS, Vol. 77, pp.244-248, published January 1980;

V: Wigler, M., et al., Cell, Vol. 16, pp.777-785, published April 1979;

W: Wigler, M., et al., Eucaryotic Gene Regulation, Proc. ICN-UCLA Symposium, R. Axel and T. Maniatis, editors, Academic Press, pp.457-475, published December 1979;

X: Wigler, M., et al., PNAS, Vol. 76, pp.1373-1376, published March 1979;

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GENE-CEN 083140

Y: Wold, B., et al., PNAS, Vol. 76, pp.5684-5688, published November 1979.

3. The invention described and claimed in the subject application was conceived by us at least as early as February 25, 1979.

4. Subsequent to our conception, we, either personally or through persons working under our direct supervision, conducted experiments in the United States reducing the invention to practice. The results of these experiments are set forth in the subject application and in various articles coauthored by us.

5. Specifically, one series of experiments involving the cotransformation process is set forth as the "First Series of Experiments" on pages 22-35 of the application. These experiments are also described in coauthored reference V, Wigler, M., et al., Cell, Vol. 76, pp.777-785, published April 1979. As this publication indicates, the manuscript was received by Cell on January 8, 1979. Therefore, it is clear that an actual reduction to practice of this embodiment of our invention was completed prior to January 8, 1979.

6. Another series of experiments involving cotransformation with multiple DNA molecules is set forth as the "Second Series of Experiments" on pages 36-45 of the application. These experiments are also described in coauthored reference Y, Wold, et al., PNAS, Vol. 76, pp.5684-5688, published November 1979. As this publication indicates, the manuscript was communicated to PNAS on July 16, 1979. Therefore, it is clear that an actual reduction to practice of this embodiment of our invention was completed prior to July 16, 1979.

7. Another series of experiments involving cotransformation is set forth as the "Third Series of Experiments" on pages 45-53 of the application. These experiments are also described in coauthored reference W, Wigler, M., et al., Eucaryotic Gene Regulation, Proc. ICN-UCLA Symposium, R. Axel and T. Maniatis, editors, Academic Press, pp.457-475, published December 1979. It is clear that an actual reduction to practice of this embodiment of our invention was completed prior to December 1979.

8. Another series of experiments involving amplification of cotransformed genes is set forth as the "Fourth Series of Experiments" on pages 54-62 of the application. These experiments were not published prior to the February 25, 1980 filing date of this application, but were subsequently published as the coauthored article, Wigler, M., et al., PNAS, Vol. 77, pp.3567-3570, in June 1980. This manuscript was communicated to PNAS on March 10, 1980. The experiments described were carried out at least as early as February 25, 1980.

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10. Another series of experiments involving transformation was carried out by us. These experiments are not described in the application and do not relate to unlinked cotransformation.

These experiments are described in coauthored reference X, Wigler, M., et al., PNAS, Vol. 76, pp.1373-1376, published March 1979. As this publication indicates, the manuscript was communicated to PNAS on December 14, 1978. Therefore, it is clear that the experiments described were completed prior to December 14, 1978.

11. As stated in preceding paragraphs 5, 6, 7 and 10, cited references V, Y, W and X, respectively, were coauthored by us. All were published less than one year prior to the February 25, 1980 filing date of this application. References V, Y and W describe embodiments of the invention conceived and actually reduced to practice in the United States prior to the publication dates of these references. Reference X describes experiments actually performed in the United States prior to the publication date of this reference.

12. Reference T, Mantei, N., et al., Nature, Vol. 281, pp.40-46 was published on September 6, 1979, less than one year prior to the February 25, 1980 filing date of this application. The Mantei, et al. paper concerns transformation with linked genes, not unlinked cotransformation. Moreover, the Mantei, et al. article does not relate to amplification of transformed genes. In addition, as clearly indicated by the second to the last paragraph of the Mantei, et al. paper, Applicants "reported cotransformation of mouse L cells with rabbit β-globin DNA and HSVI-TK DNA" in Wigler, M., et al., Cell, Vol. 16, pp.777-785, published April 1979, prior to this Mantei, et al. publication. As stated in paragraph (6) hereinabove, we actually reduced to practice the embodiment of our invention relating to cotransformation with multiple copies of transformed genes at least as early as July 16, 1979.

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We declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Nov 2 1981 — DATE — Richard Axel — RICHARD AXEL

1 — DATE — MICHAEL H. WIGLER

11-03-81 — DATE — Saul J. Silverstein — SAUL J. SILVERSTEIN

D17668-A JPW

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

12/7/81

Applicants: Richard Axel, et al.

Serial No.: 124,513 Examiner: Esther M. Kepplinger

Filed : February 25, 1980 Group Art Unit: 172

For : PROCESSES FOR INSERTING DNA INTO EUCARYOTIC CELLS AND FOR PRODUCING PROTEINACEOUS MATERIALS

30 Rockefeller Plaza
New York, New York 10112

11 November 1981

Honorable Commissioner of Patents
and Trademarks
Washington, D.C. 20231

Sir:

COMMUNICATION

Applicants submit herewith the following documents which are of interest in connection with the above-identified invention:

(a) a copy of an article from Scientific American, Volume 245, Number 1, published July 1981, entitled "Genetic Engineering in Mammalian Cells". This copy refers to work by Applicants using DNA-mediated gene transfer and is attached hereto as Exhibit A;

(b) a copy of an article entitled "Questions muddy gene transplant successes" which appeared in the September 29, 1980 issue of Chemical & Engineering News. This article which refers to work by Applicants and states: "Axel, whose team has done much to develop the method for mass movement of foreign DNA across mammalian cell membranes, has found that such cells can take up surprisingly large amounts of DNA - up to about 0.5% as much as their own DNA". This copy is attached hereto as Exhibit B;

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GENE-CEN 083145

(c) a copy of an article entitled "Gene Transfer Given a New Twist" which appeared in Science, Volume 208, published April 28, 1980. This copy which refers to use of methods developed by Applicants is attached hereto as Exhibit C; and

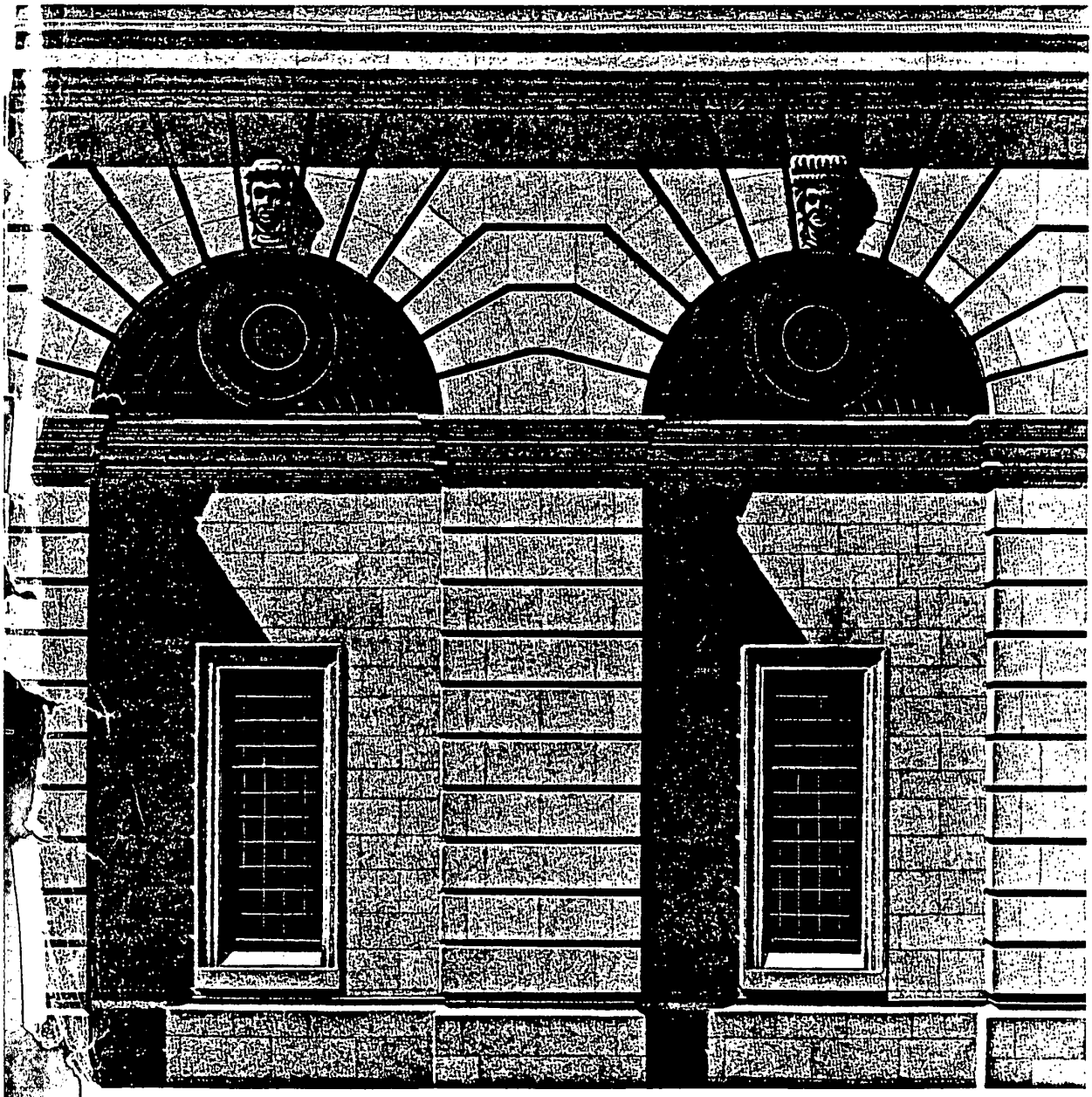
(d) a copy of an article entitled "Curing Disease With Genes" which appeared in Newsweek on April 21, 1980. This article refers to "Richard Axel of Columbia University, a pioneer in mammalian-gene transfer". This copy is attached hereto as Exhibit D.

These documents are submitted to provide the Examiner with additional information concerning the subject invention. They provide further evidence of the broad scope, pioneering nature and widespread utility of Applicants' invention. The Examiner is requested to consider these documents from this perspective and to make them of record in the subject application.

Respectfully submitted,

John P. White
Reg. No. 28,678
Attorney for Applicants
212-977-9550

SCIENTIFIC AMERICAN



HRISTOPHER WREN

Richard Axel, et al.
Serial No. 124,513
Group Art Unit 172
EXHIBIT A

$2.00

July 1981

GENE-CEN 083147

Established 1845 SCIENTIFIC AMERICAN July 1981 Volume 245 Number 1

ARTICLES

DEPARTMENTS

SCIENTIFIC AMERICAN (ISSN 0036-8733), PUBLISHED MONTHLY BY SCIENTIFIC AMERICAN, INC., 415 MADISON AVENUE, NEW YORK, N.Y. 10017.

Questions muddy gene transplant successes

At the behest of improving manipulative skills, scientists are moving genes between cells of higher organisms and back into those organisms to test the outer limits of genetic plasticity.

Yet, even as these manipulations are becoming increasingly successful—almost startlingly so—in a mechanical sense, some frustrations are developing among scientists when they deal with the question of whether such swapped genes can be made to work in their new cellular settings.

Two recent developments underscore the growing excitement and frustrations apparent in the gene transfer camp. One involves the placement of viral genes into mouse embryo cells and the subsequent stability of those genes during an embryo's maturation. The other involves successful placement of the full battery of genes responsible for biological nitrogen fixation into yeast cells. Though both achievements are important in their own right, each leaves open crucial and possibly unsettling questions.

The success with mice of Yale University geneticist Francis H. Ruddle and his colleagues Jon Gordon and George Scangos follows closely other mouse gene manipulations. They include insertion of a gene into mouse bone marrow cells and then back into mice (C&EN, April 21, page 8), and injection of specific single gene copies into mouse cells growing in tissue culture (C&EN, Oct. 15, 1979, page 6).

The latest work by the Yale team represents what might be called the "genetic overload" approach to gene transfer into mammalian cells. First, plasmids (small rings of DNA that are obtained originally from bacterial cells) are prepared containing, in addition to bacterial genes, specific virus genes. One of the virus genes, coding for an enzyme called thymidine kinase, serves as the marker card in a stacked genetic deck. Thus, it's put there not so much because it's an interesting or useful enzyme to mice, as it is one that scientists can follow with relative ease.

The plasmids are introduced into the mouse embryo cells in massive amounts—from about 1000 to 30,000 copies of the plasmid with its accompanying virus enzyme gene were made available to each mouse embryo cell. Yet even with this large-scale infusion, the cells only infrequently took up the DNA, judging from the newborn mice that still contained it. In preliminary tests, just two of 78 mice were still carrying the added DNA in their cells, Ruddle reports.

The remarkable finding is that the intruding genetic material is not thrown out of the mouse embryo cells altogether. Instead, at least in these few cases so far, some of the DNA is carried along and duplicated with the mouse's own genetic material.

Other scientists, particularly Richard Axel and his collaborators at Columbia University in New York City, have helped pave the way for Ruddle's manipulations. Axel, whose team has done much to develop the method for mass movement of foreign DNA across mammalian cell membranes, has found that such cells can take up surprisingly large amounts of DNA—up to about 0.5% as much as their own DNA.

The outsider DNA is "plunked down" at various places at the ends of mouse chromosomes, according to one scientist active in the field. Apparently enzymes in the cell "integrate" the foreign DNA with the cell's own genetic material, possibly attaching the new material at places where the chromosome has been broken. Ruddle speculated recently that the new material first passes through an unstable phase before it's integrated stably into the cell's DNA. Whatever happens, so far it's under very loose experimental control, meaning that the scientists must rely on a cell's poorly understood enzymic apparatus to accept and process the proferred DNA. This approach is "very nonphysiologic," according to one scientist.

Another approach delivers a somewhat more physiologic gene dose to a mouse cell, albeit by means of an intruding apparatus that briefly pierces through a cell's membranes into its nucleus before metering in the new genes. The microinjection apparatus and method, developed by Elaine G. Diacumakos of Rockefeller University in New York City, was used by W. French Anderson and his colleagues at the National Institutes of Health in Bethesda, Md., to bring foreign genes into cultured mouse cells, in experiments first made public a year ago.

Like Ruddle's team, the NIH group uses DNA in the form of a plasmid. But unlike the Yale group, Anderson and his colleagues introduce that plasmid into a mouse cell in a minuscule instead of a massive dose: essentially as a "single copy," Anderson says. That is, the plasmid is treated just like any chemical solution and is diluted appropriately so that a single copy is injected into a single

mouse cell, held in place, and observed under a microscope.

The injected single copy acts very differently from the way the massively introduced material behaves, Anderson continues. "We see free, extrachromosomal plasmids that can be rescued back out of cells even after multiple generations of cell growth," he says. Exactly what is going on is not particularly clear, he adds, but the material acts unlike the integrated DNA described by Ruddle. There are hints that some copies of the microinjected DNA also might be integrated into the recipient cell's chromosome. Nonetheless, at least one copy is carried by such cells as an intact, circular plasmid.

That's remarkable for several reasons. One is that mammalian cells normally do not carry plasmids, which after all are DNA rings that have been purloined from microbial cells. Thus their presence and continued tolerance in mammalian cells is unusual, to say the least. But the plasmids are remarkable for still other reasons. During their stay in the mouse cells, which reproduce in vitro, the plasmids undergo genetic recombination, following an unexpected pattern, Anderson says.

Recombination is a natural process whereby similar but distinct bits of genes are exchanged from one molecule to another. For example, arms of duplicate chromosomes can be interchanged, typically through a particular region. Anderson says that the interloping plasmid DNA also can go through this process repeatedly in the mammalian cell, and that one particular region of the plasmid is a "hot spot" for recombination. Thus, something about this foreign bit of DNA is familiar to a mouse cell's recombination apparatus.

Despite this interesting and seemingly successful gene-introduction phenomenology, a key problem is beginning to nag many of the scientists doing this research. Will the genes work once they're placed into the mammalian cells? The answer is crucial to the clinical future of such experiments where the eventual goal is to correct certain genetic defects, such as various debilitating anemias (see page 42) and other enzyme deficiency diseases.

The underlying strategy with this is to replace or to augment inadequate genes with test-tube-improved variants. And, although some people fear such genetic manipulation is prone to misuse, the scientists now working toward it see themselves laboring on behalf of patients whose current hopes for a near normal life are extremely slim and depend on lifelong reliance on various drugs.

So far, the genes introduced artificially into mammalian cells work only fitfully, at best. Relatively unspecialized enzymes having what one scientist terms "housekeeping duties" seem to function more successfully than do more specialized gene products, such as the globin proteins that make up the oxygen-carrying hemoglobin molecule in blood.

Here the central problem is that scientists have few clues on how such specialized genes are regulated. There are a variety of technical tricks already available to get some of these genes to work minimally. But the big question remains whether that low activity can approximate the body's needs in a reasonable way. Normally, for instance, hemoglobin is made only in red blood cells, and there in huge amounts. Few researchers can be coaxed into thinking that it will be satisfactory either to make small amounts of hemoglobin in every cell of the body or to make huge amounts everywhere.

Current findings indicate that whatever controls the hemoglobin gene is not present or is not working properly in some very large DNA pieces that are attached to the hemoglobin gene proper and that have been moved into mouse cells, Anderson says. This may mean that the whole question of [illegible] portant gene [illegible] to be enti[illegible] plicated t[illegible] far. But t[illegible] phisticate[illegible] yet have [illegible]

A simil[illegible] scientists [illegible] that are equally crucial in nature, but in the plant instead of the animal kingdom. Aladar A. Szalay and Ada Zamir (a visiting scientist from Israel) working at Cornell University, Ithaca, N.Y., have put the genes controlling biological nitrogen fixation into yeast. The team also includes yeast geneticist Gerald R. Fink, who has helped develop reliable methods for moving genes into and out of yeast cells.

"We do not know anything yet about expression [function] of the genes in yeast," Szalay says. "We're just beginning to look."

The situation is unusual because the Cornell team has introduced so many genes deliberately at once into a recipient cell. (Thus it's different from the work where one or a few genes are introduced singly or in multiple copies into mouse cells.) The more than a dozen genes necessary for nitrogen fixation are carried on a really big-sized piece of DNA, containing about 45,000 nucleotide bases (this is roughly 1% of the entire DNA

Continued on page 42

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Continued from page found in a bacterium, such as *Escherichia coli*).

"It's amazing this big piece is stable in yeast," Szalay says. The whole piece is integrated into a yeast chromosome and is carried along (and replicated) during the yeast's normal growth cycle for many generations. The nitrogen-fixation genes are preserved when yeast cells undergo meiosis, during which duplicate copies of genetic material are halved in preparation for the sexual reproduction side of the yeast life cycle.

The available means for manipulating such complex packages of genes are more sophisticated for yeast cells than for mammalian cells. The plasmids that carry the nitrogen fixation genes are, in effect, programed to integrate into the yeast chromosome. The Cornell team's work is even a bit more fanciful than that, involving a kind of of flying trapeze effect, during which one set of genes on a plasmid was used to swing into the chromosome another set of genes on a second plasmid.

Regardless of such high-flying techniques, the question remains whether the genes can work inside the yeast cells. And, the answer to that question is the premise for going on to the eventual challenge of transferring those genes into green plants, such as corn, that currently depend on an expensive outside supply of nitrogen fertilizer.

"If we can't get expression [of the nitrogen fixation genes] in yeast, it's not worth spending the public's money to get it to work in plants," Szalay says. But he's confident that "we can push yeast to do it."

Jeffrey Fox, Washington

Iron chelators may aid in anemia treatment

The National Institutes of Health's six-year-old project to develop a new drug for use in treating patients with the genetic blood disease Cooley's anemia is coming up with some unexpected findings. As its initiators had hoped, the project is beginning to produce some very promising drug candidates.

But it also is showing both academic researchers and governmental administrators that the task of developing a successful drug is an enormously complicated one in which successful chemistry is by no means the only critical factor.

The project's aim is to develop a safe, effective, inexpensive, and easy-to-take drug to remove the excess iron that builds up in patients with Cooley's anemia (C&EN, May 1977, page 24). These people produce inadequate amounts of the beta chain of the hemoglobin molecule so that their own blood is not an effective oxygen carrier. They require regular blood transfusions to survive, but this treatment eventually overloads their bodies with iron. The iron accumulates particularly in the kidneys, liver, and heart, where it leads to organ malfunction and death, often when the patient is in the mid-teens or early twenties. The current project does not aim at curing Cooley's anemia, but rather at controlling a potentially fatal side effect—the buildup of iron in critical tissues.

An early success of the program, most involved in it agree, was getting a drug approved for removing excess iron from Cooley's anemia patients. This was accomplished in 1977 when deferrioxamine, a drug used for treating acute iron poisoning, was approved for use in this disease.

Deferrioxamine is not an ideal drug, however. It must be given by injection, and, since its half-life in the body is very short, it works best when it is slowly infused for six to 12 hours each day. Such treatment is prohibitively expensive: Elmer B. Brown, professor of medicine at Washington University school of medicine in St. Louis, and a participant in the NIH project, estimates that drug costs alone are more than $4500 per year for this therapy.

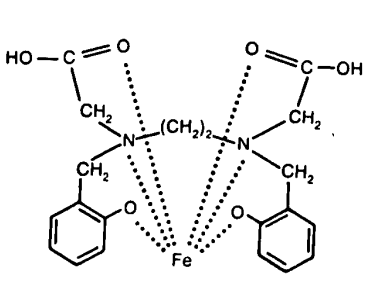
However, better drugs may be on the way. The one that is presently causing the most excitement is being developed by Colin G. Pitt, a chemist at Research Triangle Institute in North Carolina. Pitt has several compounds that remove more iron from rat and mouse models than does deferrioxamine. His most promising compounds are ester derivatives of *N,N'*-bis(2-hydroxybenzyl) ethylenediamine-*N,N'*-diacetic acid or HBED. This compound itself is a better remover of iron from these animal models than is deferrioxamine. However, when it is made into the dimethyl, dipropyl, or dipentyl ester derivative, it becomes even better. These ester derivatives are orally active and less toxic than HBED in acute toxicity tests in animals, but like HBED they are better than deferrioxamine at removing iron from animals. The derivatives are an order of magnitude better at removing iron from the animals than anything previously tested, Pitt says. In fact, they are able to remove nearly 30% of the iron theoretically available in the animal, he says.

"If the efficacy of the esters of HBED is confirmed clinically, the goal of identifying an orally active chelating agent capable of controlling iron overload will have been achieved," Pitt told a symposium on the development of iron chelation drugs held in San Francisco last month.

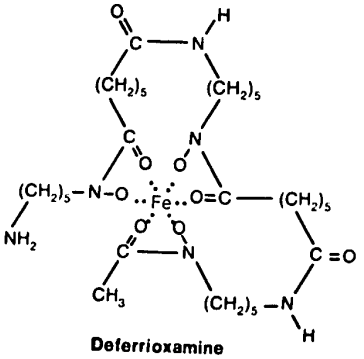
Finding these compounds was a combination of logical chemical investigation and pure luck, Pitt points out. The compounds are phenol derivatives, a class of compounds expected to bind ferric ion tightly based on the stability constants of iron(III) complexes of phenol. Furthermore, many bacteria contain phenolate compounds called siderophores, which the bacteria produce in order to scavenge iron from their environment. In the molecule HBED, carboxylate and amino groups also are present to assist the compound in binding iron. These groups, too, often are found in naturally occurring siderophores.

However, the chemical behavior of these compounds is not completely predictable from their structures and the stability constants of their iron complexes. Pitt and colleagues tested many compounds expected to have a high affinity for iron based on these criteria, and found them to be inac-

HBED shows promise as replacement for deferrioxamine in treating Cooley's anemia



HBED
(*N,N'*-bis(2-hydroxybenzyl)ethylenediamine-*N,N'*-diacetic acid)



Deferrioxamine

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particles and their behavior in order to deduce a flow velocity and direction.

The Voyager radio-wave sensor, much to the surprise of its designers, made some observations that may tip the balance on the corotation controversy. Joseph Alexander and Michael Kaiser of Goddard Space Flight Center, Greenbelt, Maryland, discovered a new type of radio emission from Jupiter's magnetosphere. And the source region seems to circle the planet 3 to 5 percent slower than the planet spins. Kaiser and Alexander think that the signal comes from the outer edge of Io's doughnut-shaped cloud. They conclude that the plasma that is producing the radio waves in this region is not quite keeping up with the planet's rotation—in agreement with Bridge's measurements of the flow velocity in the same area.

Krimigis's data indicate that the Jovian merry-go-round does not go on forever. On the nightside of Jupiter, between 9 million and 10 million kilometers from the planet, "the plasma can't hang on anymore—it streams off away from Jupiter." This flow, which Krimigis dubs the magnetospheric wind, may resemble the solar wind, a flow of particles away from the sun. By studying in detail the Jovian magnetospheric wind, Krimigis hopes to understand the physics that drives it and, by analogy, the solar wind and the winds emanating from many other stars.

While the two Voyagers and the two Pioneer spacecraft that preceded them have revealed much about the Jovian magnetosphere, many of the new findings are surprises or cannot be fathomed completely with available data. Scientists eagerly await the Galileo spacecraft, "may it fly," to test their speculations about how material from Io's volcanoes is distributed into the magnetosphere and to resolve the corotation controversy. Galileo is supposed to orbit Jupiter after it is launched in the mid-1980's by the problem-plagued Space Shuttle.

—BEVERLY KARPLUS HARTLINE

Richard Axel, et al.
Serial No. 124,513
Group Art Unit 172

EXHIBIT C

Gene Transfer Given a New Twist

The demonstration that genes introduced into bone marrow cells work in living mice is the most recent development in a series of advances in gene transfer.

Biology has seen so many surprises in the past few years that the unexpected is becoming commonplace. Still, reports on television and in the newspapers that a "revolutionary" method of gene transfer had been discovered surprised many people, among them a number of scientists who are themselves studying methods of gene transfer between cells.

The reports described experiments in which a group of investigators* at the University of California at Los Angeles (UCLA) successfully introduced new genes into mouse cells and showed that the genes appeared to work when the cells were put back into living mice. Now this, in outline, is just what genetic engineering is all about.

Investigators have long sought a practical method of introducing new, functional genes into living organisms because such a technique might permit gene replacement therapy—a true cure for diseases, sickle-cell anemia, for example, which are caused by a single defective gene. And so the UCLA results were newsworthy.

Some news reports overstated their import, however, possibly aided by a press release from the university that hailed the techniques as "revolutionary," a word that subsequently appeared in the headline of a Washington *Post* story on the new developments. A more circumspect description was given by one researcher, who requested that he not be identified. "It is a nice experiment," he said, "but a logical extension of previous research." In other words, it fell something short of revolutionary.

In fact, investigators have been transferring genes between mammalian cells for years by a variety of techniques. Some of the earlier methods used cell fusion to produce hybrids bearing whole chromosomes or chromosome pieces from both cell types. More recently, investigators, with the aid of recombinant DNA technology, have devised more specific procedures for introducing individual genes into cells. During the past year and a half, for example, three groups of investigators reported the introduction of a globin gene from one animal species into cells from another. (Globin is the protein portion of the hemoglobin molecule.) In at least two of these cases, the transferred gene expressed itself in the synthesis of the appropriate globin protein.

The UCLA achievements grew out of this previous gene transfer work. According to Martin Cline, leader of the research group reporting the results, "We used established techniques for transferring the genes, but took them one step further—to an in vivo system."

In one series of experiments, described in the 3 April issue of *Nature*, the investigators induced resistance to the drug methotrexate in bone marrow cells by incubating them with DNA prepared from a line of methotrexate-resistant mouse cells. Methotrexate, which is used for cancer chemotherapy, kills cancer cells by inhibiting the enzyme dihydrofolate reductase (DHFR) and thus preventing the synthesis of the essential chemical folic acid.

Cancer cells may become resistant to methotrexate (or other cancer drugs, for that matter), in which case they are no longer killed by the drug, at least in concentrations that are tolerated by the rest of the body. Work from the laboratory of Robert Schimke, who is also at UCLA, has shown that methotrexate resistance is sometimes caused by amplification of the DHFR gene. Resistant cells, having many more copies of the gene than nonresistant ones, produce so much of the enzyme that they can live even in the presence of the drug.

The Cline group prepared DNA from methotrexate-resistant mouse cells having many extra copies of the DHFR gene. For the DNA preparation and subsequent gene transfer, they used techni-

*Martin Cline, Howard Stang, Karen Mercola, L. Morse, R. Ruprecht, Jeffrey Browne, and Winston Salser.

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ods developed in the laboratory of Richard Axel, of Columbia University College of Physicians and Surgeons, that are themselves based on procedures devised by Silvia Bacchetti and Frank Graham of McMaster University in Hamilton, Ontario.

The DNA preparation was then incubated with bone marrow cells taken from a strain of mice whose cells all bear a distinctive and easily recognizable marker chromosome. During the incubation, some of the cells took up the DNA, a process called transformation. (This kind of transformation, simply meaning the acquisition of new genes, is different from the malignant transformation by which normal cells become cancerous.)

One of the major problems in gene transfer experiments is identifying the cells bearing the desired new gene. Drug resistance provides a solution to the problem because only cells with the resistance gene can multiply in the presence of the drug and, consequently, they can be selected out of a mixture of cells, most of which lack the resistance gene.

Usually selection is carried out on cells in culture. And it is here that the Cline group added a new twist to gene transfer because they injected the transformed bone marrow cells, together with an equal number of "mock transformed" cells, back into living mice for the selection procedure. Cells were mock transformed by incubating them with DNA not containing multiple DHFR genes; they did not carry the chromosome marker and so could be distinguished from the transformed variety. The mice, which were genetically and immunologically compatible with both types of injected cells, had been previously irradiated to destroy their own bone marrow.

To select for resistant bone marrow cells, Cline and his colleagues then treated the mice with methotrexate. This drug, like most of the others used for cancer therapy, may also kill normal cells, especially those, including bone marrow cells, that divide rapidly. The methotrexate dose was carefully chosen to depress the division of nonresistant bone marrow cells without being so high that it killed the mice.

Under this selective pressure, the bone marrow of the animals became enriched with cells bearing the marker chromosome—that is, with cells that had been transformed by exposure to DNA carrying multiple DHFR genes. This result indicates that the methotrexate-resistance genes entered the cells and were working there. In support of this hypothesis, Cline points out that cells taken from the animals had two to four times as much DHFR activity as cells taken from controls that had not received transformed cells. In addition, the blood status of methotrexate-treated mice that had been injected with transformed cells was better than that of drug-treated controls. For example, the former had a higher percentage of red cells in their blood than the latter.

All in all, the evidence strongly suggests that genes for methotrexate resistance were transferred into the bone marrow cells, but a direct demonstration of the presence there of the transferred genes was not possible. The DHFR genes obtained from methotrexate-resistant mouse cells are not distinguishable from those already present, although in smaller numbers, in all mouse cells, Cline explains.

> "Expression in inappropriate tissue or abnormal control in the cell in which they [the transferred genes] are normally expressed could be disastrous."

For this reason, the UCLA workers undertook a second series of experiments in which they transferred a viral gene, the thymidine kinase gene of herpes simplex virus, into murine bone marrow cells. Because the viral DNA can be distinguished from that of mice, the investigators could detect the viral material in the transformed cells, thus obtaining direct evidence for gene transfer. In these experiments, as in the case of the DHFR gene transfer, the investigators selected for the cells bearing the viral thymidine kinase gene in living mice. A description of the thymidine kinase work is currently in press at *Science*.

Although the UCLA group has taken another step on the road to a practical method for genetic engineering, a number of barriers, some formidable, will have to be surmounted before there is a cure for sickle-cell anemia or any other genetic disease. "The principal obstacle," says Cline, "is the need to control the expression of the gene."

Simply transferring the gene and finding a way to select for the cells bearing it is not enough. The gene product must also be made only in the appropriate cells, at the right time, and in the correct amount. Bob Williamson of St. Mary's Hospital Medical School in London, writing in the News and Views section of the same issue of *Nature* in which the UCLA work was reported, says, "Expression in inappropriate tissue or abnormal control in the cell in which they [the transferred genes] are normally expressed could be disastrous." Unfortunately for genetic engineers, gene control in mammalian cells is one of the bigger mysteries remaining to molecular biologists.

One manipulation that might help to bring a transferred gene under appropriate control is to introduce it into the chromosome at the site where it normally resides. At present, there is no way to direct a gene to a specific chromosomal site or to ensure that it is integrated into a chromosome at all. It is even difficult to determine whether or not a transferred gene has been integrated, and this has not yet been accomplished for the DHFR and thymidine kinase genes transferred by the UCLA group.

Cure of genetic diseases is not the only potential application of genetic engineering, however. Cline suggests that the techniques developed at UCLA might be of value in cancer chemotherapy. The harmful effects on the bone marrow of many anticancer drugs often limit the treatment received by a cancer patient. Cline's idea is to remove bone marrow cells from a patient who is about to undergo chemotherapy. After they are transformed by genes conferring resistance to whatever drug is to be used, the cells can be given back to the patient. If these transformed cells establish themselves in the patient's bone marrow, as the methotrexate-resistant cells did in the mice, then the patient might be able to tolerate higher doses of the drug or a longer course of treatment with it than might otherwise be possible. Cline suggests that an application such as this might be tested in human patients within "3 to 5 years." Time, as they say, will tell.—J. [illegible]

Court of Customs and Patent Appeals

In re Strahilevitz

No. 81-563
Decided Jan. 15, 1982

PATENTS

1. Pleading and practice in Patent Office — Rejections (§54.7)

Specification — Sufficiency of disclosure (§62.7)

PTO's burden of proof in calling into question enablement of appellant's disclosure requires that PTO advance acceptable reasoning inconsistent with enablement; thereupon, burden shifts to appellant to show that one of ordinary skill in art could have practiced claimed invention without undue experimentation.

2. Specification — Sufficiency of disclosure (§62.7)

Although working examples are desirable in complex technologies and detailed examples can satisfy statutory enablement requirement, examples are not required to satisfy Section 112, first paragraph; therefore, examiner's statement that "nearly universal applicability" alleged for invention necessitated numerous examples was erroneous.

3. Specification — Sufficiency of disclosure (§62.7)

Patents that issued after filing date of applicant's parent application cannot be relied upon as evidence of enablement by applicant who wishes to retain benefit of parent application's filing date.

4. Court of Customs and Patent Appeals — Issues determined — Ex parte patent cases (§28.203)

On appeal to Court of Customs and Patent Appeals, Solicitor cannot raise new ground of rejection or apply new rationale to support rejection affirmed by Board of Appeals.

Particular patents — Hapten Removal

Strahilevitz, Immunoassay and Treatment Methods, rejection of claims 36-48 reversed.

Appeal from Patent and Trademark Office Board of Appeals.

Application for patent of Meir Strahilevitz, Serial No. 761,290, filed Jan. 21, 1977, continuation of application, Serial No. 255,154, filed May 19, 1972. From decision rejecting claims 36-48, applicants appeal. Reversed.

J. Philip Polster, St. Louis, Mo., for appellant.

Joseph F. Nakamura and Fred W. Sherling for Patent and Trademark Office.

Before Markey, Chief Judge, and Rich, Baldwin, Miller, and Nies, Associate Judges.

Miller, Judge.

The decision of the Patent and Trademark Office ("PTO") Board of Appeals ("board") sustaining the rejection of claims 36-48[1] as unsupported by an adequate disclosure required by 35 USC 112, first paragraph, is *reversed*.

Background

Invention

Appellant's invention relates to methods and devices for removing a hapten, antigen, or antibody from the blood of a living mammal.

A hapten is a small molecule which does not by itself produce antibodies but which, when conjugated to a carrier protein or other macro-molecular carrier, induces in a recipient animal or human the production of antibodies that are specific to the small molecule. For example, certain psychoactive drugs, such as LSD, heroin, and tetrahydrocannabinol, can function as haptens. When an antibody (a relatively large immunoprotein) contacts the hapten or antigen to which it is specific, it tightly binds the hapten or antigen. It is this specific binding property of antibodies which is used by appellant to remove haptens, antibodies, or antigens from blood. Claim 44, the broadest appealed claim, from which claims 45 and 46 depend, reads:

> An immunological method for removing from a living mammal a hapten in the blood of said mammal, comprising connecting in the blood circulatory system of said mammal a hapten-removing device, said device comprising passage means for said blood; an antibody to said hapten in

[1] Application serial No. 761,290, for "Immunoassay and Treatment Methods, Particularly of Psychoactive Drugs," filed January 21, 1977; continuation of serial No. 255,154, filed May 19, 1972.

Richard Axel
Serial No. 124,513
Group Art Unit 172

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EXHIBIT D

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said device; and exposure means in said device for exposing said hapten to said antibody and for preventing said antibody from entering said circulatory system.

Appealed method claims 36-39 involve linking to a matrix an antibody to a hapten and passing blood over the matrix. The antibody removes the hapten from the blood by binding the hapten.[2] Claim 40 is directed to a method for removing an *antibody* from the blood of a living mammal which is passed over an *antigen* linked to a matrix; claim 41 involves an antibody linked to a matrix to remove an *antigen* from blood.

Claims 42 and 43 involve a method for removing a hapten or an antigen from blood through an immunodialysis process.[3] A semipermeable membrane, permeable to haptens or antigens and impermeable to antibodies, separates a patient's blood from a solution containing antibodies to the target molecules. Because the antibodies specifically bind target molecules that diffuse from the patient's blood through the semipermeable membrane, a gradient across the membrane is present for the molecule, which continues to diffuse from the blood to the dialysis solution as long as the solution contains antibodies which are free to bind the target molecules. Claims 47 and 48 are essentially directed to devices for practicing claims 42 and 43.

Proceedings Below

The examiner rejected the claims as based upon an insufficient disclosure under 35 USC 112, first paragraph, stating:

> The disclosure is essentially an invitation to experiment. No specific examples are given. No human treatment (in fact no animal treatment) is described. The specification is replete with statements as to what *may* be done. No dialysis or adsorption data [have] been presented.
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>
> * * * [A]ppellant urges "nearly universal applicability" for *selectively* removing chemical species. If there is in fact universal applicability, with selectivity, appellant should have inserted numerous (50 to 100 for instance) examples into the specification.
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> * * *
>
> Appellant urges that no working example is required and that instructions to a technician are not required. But it has already been pointed out that appellant believes his device has *universal* applications (and the claims are just about that broad). Admittedly, one skilled in the art is a Ph.D., but even with such a high level of skill, such a person would need detailed guidance to practice the claimed alleged *universal* invention.

The board took a somewhat different approach:

> [A]ppellant admits * * * the disclosure contains no "operative example". While we recognize that specific examples are not necessary to meet the requirements of Section 112, In re Gay, 50 CCPA 725, 309 F.2d 769, 135 USPQ 311 (1962), when present, they do provide good evidence that the disclosure is enabling and that the invention may be performed without undue experimentation. In our view, the

[2] Claim 36 reads:

> An immunological method for removing from a living mammal a hapten in the blood of said mammal, comprising a step of connecting in the blood circulatory system of said mammal a hapten-removing device, said device comprising inlet means and outlet means for connecting said device into said circulatory system, chamber means for defining a flow passage between said inlet means and said outlet means, a matrix in said chamber, and an antibody to said hapten linked to said matrix, said antibody binding said hapten to said matrix as said blood passes through said chamber, and a further step of separating said hapten from said antibody to regenerate said antibody for further use in said device.

[3] Claim 42 reads:

> An immunological method for extracting from a living mammal a hapten in the blood of said mammal, comprising
>
> (1) connecting in the blood circulatory system of said mammal a hapten-removing device, said device comprising a first chamber and a second chamber; a semipermeable membrane separating said first chamber from said second chamber; inlet means and outlet means for connecting said first chamber in the circulatory system of said mammal; a liquid phase containing antibodies to said hapten in said second chamber, said membrane being permeable to said hapten and being impermeable to said antibodies; and closed regenerative means connected in a fluid circuit with said second chamber, said regenerative means comprising a third chamber,
>
> (2) drawing at least a portion of said liquid phase from said second chamber to said third chamber,
>
> (3) changing the pH of said liquid phase in said third chamber for releasing said antibody from said hapten, and
>
> (4) returning said antibody to said second chamber and preventing said hapten from returning to said second chamber.

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material most descriptive of the claimed methods and apparatus, is that presented in examples 12 and 13, wherein systems are defined for the removal of haptens, antigens and antibodies from the circulatory system. At best, the descriptive material is a generalized explanation of figures 6 and 7 in the drawings, and except for the regeneration step, provides no specific or even general guidelines for the treatment. The claimed methods * * * involve a complicated and complex series of reaction conditions that apparently require specifically related haptens, antigens and antibodies to operate effectively. We fail to find in the disclosure adequate instruction as to how these selections are to be made and in our view such selections would not be readily apparent or obvious to those of ordinary skill in the related art. Also required in various claims is the use of a matrix for use in binding the antibodies; however, we see no disclosure adequate to define such production or even the materials that are compatible in use. The specification also fails to teach or even suggest the antibodies or antigens that may be used with the appropriate haptens and we see no disclosure that would lead to or suggest a proper means of selecting the appropriate membranes for use in the removal devices. In short, it appears that the appellant has developed a concept of how a variety of prior art procedures may possibly be combined in a single method * * * and then retreated to leave those in the art with the task of experimentation to see if the method can be made to operate as set forth. * * * Here, appellant has presented no descriptive material that in our view is sufficient to provide a proper instruction in the manner of either developing the apparatus as claimed or the method of using same. The wide variety of variables that are inherent in the process, and for which the disclosure provides no basis for evaluation, leads us to the conclusion that operation of the method as claimed would involve an undue amount of experimentation and we will therefore sustain the rejection.

Thus, the board questioned whether the specification enabled selection and preparation of haptens, antigens, and antibodies; preparation of the matrix; selection of the dialysis membranes; and selection of parameters of the dialysis process.

Opinion

Burden of Proof

[1] A threshold issue is whether the PTO met its burden of proof in calling into question the enablement of appellant's disclosure. This burden required that the PTO advance acceptable reasoning inconsistent with enablement. Thereupon, the burden would shift to *appellant* to show that one of ordinary skill in the art could have practiced the claimed invention without undue experimentation. In re Sichert, 566 F.2d 1154, 1161, 196 USPQ 209, 215 (CCPA 1977).

[2] The examiner reasoned that because of the breadth of the invention, a large number of examples (50 to 100) would be *required* to enable one of ordinary skill in the art to make and use the invention. 35 USC 112, first paragraph. We recognize that working examples are *desirable* in complex technologies and that detailed examples can satisfy the statutory enablement requirement. Indeed, the inclusion of such examples here might well have avoided a lengthy and, no doubt, expensive appeal. Nevertheless, as acknowledged by the board, examples are not *required* to satisfy section 112, first paragraph. See, e.g., In re Stephens, 529 F.2d 1343, 188 USPQ 659 (CCPA 1976); In re Borkowski, 57 CCPA 946, 422 F.2d 904, 164 USPQ 642 (1970); In re Gay, 50 CCPA 725, 309 F.2d 769, 135 USPQ 311 (1962). Therefore, the examiner's statement that the "nearly universal applicability" alleged for the invention necessitated numerous examples was erroneous. Although the invention is applicable to a large variety of haptens and antigens, the examiner offered no reason why these different compounds would require different techniques or process parameters.

However, the examiner (and the board) also reasoned that enablement was not present because no dialysis or adsorption (of the antibody or antigen to the matrix) data were presented, and we are persuaded that this was sufficient to shift the burden to appellant to establish that a person of ordinary skill in the art could have practiced the invention without undue experimentation.

Enablement

The dispositive issue is whether appellant's disclosure, considering the level of ordinary skill in the art as of the date of appellant's application, would have enabled

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GENE-CEN 083156

a person of such skill to make and use appellant's invention without undue experimentation.

[3] Appellant explains that his invention resides in combining the known prior art techniques of hemodialysis or hemoperfusion with immunochemical dialysis and immunochemical adsorption. He properly relies on literature citations to establish both the level of ordinary skill in the art and the fact that the techniques necessary to practice his invention were known in the art.* In re Eynde, 480 F.2d 1364, 178 USPQ 470 (CCPA 1973).

A. Selection and Preparation of Haptens, Antigens, and Antibodies

Appellant argues that methods for forming antibodies which are specific to particular haptens and antigens were well known prior to his filing date. In his specification, he states:

> Immunization of rabbits with conjugates in complete Freund's Adjuvant are carried out by a similar procedure to the one described by Strahilevitz et al, supra. The preparation of antisera and globulin fractions are also described in this reference. The antibodies may be purified by known methods such as those set out in Immunologic Methods in Steroid Determination.

He then gives a specific example of preparing an antibody to tetrahydrocannabinol, referring to a method described in Immunologic Methods in Steroid Determination (Peron & Caldwell ed. 1970) for conjugating the compound to a protein, and detailing the injection of "[r]abbits, sheep or other suitable animals" with the conjugate, giving concentration, dosage, and frequency of injection data.

Pinckard & Weir, Equilibrium Dialysis and Preparation of Hapten Conjugates in Handbook of Experimental Immunology 493 (D. Weir ed. 1967), cited in appellant's specification and made a part of the record in the parent application, details the preparation of protein-hapten conjugates, injection of the conjugates into animals, and recovery of antibodies to the haptens from the animals. It is clear from this disclosure that selection and preparation of related haptens, antigens, and antibodies involve routine and well-known techniques. Indeed, at oral argument, the Solicitor acknowledged that techniques for preparing antibodies to specific haptens and antigens were known in the art.

B. Preparation of Matrix with Bound Antibodies

In example 13 of appellant's specification, appears the following statement:

> As shown in Figure 7, the apparatus for this method consists of a column 26 which includes a matrix 37 to which a binding species is linked. The binding species is a hapten, antigen (including a hapten conjugated to a carrier) or antibody which reacts specifically with the species which is to be removed from the blood. The linkage of the binding species to the matrix 37 may preferably be directly to the matrix, as when the matrix is made of a synthetic polymer such as polystyrenelatex. The linkage may also be through a suitable solid phase coating on the matrix. The antibody is then linked to the coating by one of the known methods for the preparation of immunoadsorbents, for example by a modification of one of the methods of Campbell (Campbell et al, Proc. Nat. Acad. Sci., U.S.A., 37, p. 575 (1951); Malley and Campbell, J. Am. Chem. Soc. 85, p. 487 (1963)). If any chance exists that the solid phase adsorbent may break loose from the matrix 37, suitable filters are necessary in the system. The matrix may simply be the wall of the (plastic) column if the length of the column is sufficient to provide the required surface area for interaction at the blood-binding species interface.

Additionally, appellant points out that appropriate matrices and techniques for binding antigens or antibodies thereto were well known in the art, as evidenced by Weetall, U.S. Patent No. 3,652,761, cited by the examiner in support of a rejection under 35 USC 103 which was reversed by the board. Weetall discloses that it was known in the art to couple antigens to cellulose and its derivatives, polyaminopolystyrene, dextrans, and polyaminoacids to remove antibodies from serum. This patent also teaches a novel method for coupling an-

* Some of the patents cited by appellant to show the level of ordinary skill in the art issued after the May 19, 1972, filing date of his *parent* application. These patents, originally cited by the examiner as section 102(e)/103 prior art against the claims, cannot be relied upon as evidence of enablement if appellant wishes to retain the benefit of his parent application's filing date. See In re Gunn, 537 F.2d 1123, 190 USPQ 402 (CCPA 1976), and In re Budnick, 537 F.2d 535, 190 USPQ 422 (CCPA 1976). However, as will be developed infra, reliance upon these patents is unnecessary.

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tigens or antibodies to glass with the use of a silane coupling agent.

C. Dialysis Membranes

The position of the board and the Solicitor is that appellant has provided no disclosure regarding selection of the dialysis membranes required to practice the subject matter of claims 42, 43, 47, and 48. However, appellant's specification states:

> Such membranes, having various pore sizes, and thus which are permeable to molecules of various sizes are commercially available . . . . The semipermeable membrane is chosen to be of such a porosity and permeability as to be permeable to small molecules like the intoxicating hapten of interest, but is not permeable to large molecules present in the blood of the patient such as serum proteins.

Selection of the membranes, therefore, is based upon their porosity and the relative molecular sizes[1] of the antibody and the hapten or antigen. Weetall, supra, teaches that antigens generally have a molecular weight of 10,000 or higher, and that antibodies have molecular weights of 160,000 to 1,000,000. Haptens are much smaller.[2] Galletti et al., in U.S. Patent No. 3,619,423, disclose a process in which enzymes in a dialysis fluid remove undesirable substances from blood. The blood is separated from the dialysis fluid by a semipermeable membrane having a pore size which will pass compounds having a molecular weight of less than 10,000. The 100,000 molecular weight enzymes cannot cross the membrane. Thus, these references clearly indicate that selection of semipermeable membranes on the basis of pore size, as suggested by appellant's specification, was a technique known in the art prior to appellant's filing date.

D. Dialysis

Appellant states that a person of ordinary skill in the art would know appropriate flow rates and other parameters for performing dialysis, citing Galletti et al., supra, and Rosenbaum, Kramer, Raja, & Boreyko, Resin Hemoperfusion, 284 New England J. Med. 874 (1971). Galletti et al. teach a hemodialysis method which uses enzymes rather than antibodies to remove materials from blood. Dialysis parameters are disclosed, including a flow rate of 200 ml./min. Rosenbaum et al. teach a method in which blood is passed through a column at 300 ml./min. for three hours to remove barbiturates from the blood. These references disclose typical dialysis and related data in methods similar to those of appellant. There is no reason to believe that these parameters would not also be applicable to appellant's methods, as he contends.

[4] In view of all the foregoing, we hold that appellant's disclosure would have enabled a person of ordinary skill in the art to make and use appellant's invention without undue experimentation.[3]

The decision of the board is *reversed*.

Reversed.

[1] Molecular size is a function of molecular weight.

[2] For example, heroin, LSD, and tetrahydrocannabinol all have molecular weights of between 300 and 400.

[3] The Solicitor has not seriously attempted to refute appellant's positions on the enablement questions raised by the board and the examiner, on the proper interpretations of the references, or on the applicability of the references to the practice of the claimed invention. Instead, the Solicitor raises a new argument by challenging the *operability* of the invention in removing antigens or haptens from whole blood. However, it is well settled that, on appeal to this court, the Solicitor cannot raise a new ground of rejection or apply a new rationale to support the rejection affirmed by the board. In re Armbruster, 512 F.2d 676, 185 USPQ 152 (CCPA 1975). Even if we were to consider the Solicitor's new argument, we would have to reject it, because the Solicitor has shown no reason for doubting appellant's statements, in his specification, that the process will remove material from whole blood as stated. Indeed, the references cited by the Solicitor which show antibodies removing antigens or haptens from serum are convincing that appellant's process will operate with whole blood.

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SCIENCE

Curing Disease With Genes

Ever since scientists began tinkering with genes, they have promised that they would cure genetic diseases ranging from diabetes to dwarfism. Last week, they began to make good on that promise. Researchers at the University of California, Los Angeles, disclosed that they had successfully transferred genes for the first time into living animals—in this case, mice. "This technique will allow us to treat genetic diseases and insert desirable genes into plants and animals," predicts Martin J. Cline, who led the research team. "The possibilities are enormous."

The UCLA experiment exploited the technology of recombinant DNA, which enables researchers to transfer genes from one organism to another (NEWSWEEK, March 24); heretofore, it has been accomplished only in cell cultures. In the first step, biologists Cline, Howard Stang, Karen Mercola, Winston Salser and others isolated genetic material from mice, including the gene that confers resistance to an anti-cancer drug. The scientists extracted many copies of this gene and mixed them with calcium to produce tiny gene-containing crystals. The microcrystals were then mixed with bone-marrow cells from other mice. Some of the new cells engulfed the microcrystals and consequently picked up the trait of drug resistance. These hybrid cells were then injected into the veins of compatible mice. Finally, the scientists ran tests to prove that the new gene was working in the bone-marrow cells.

The first spin-off of the research will probably come in cancer therapy. Methotrexate, a common chemotherapy drug, kills tumors but also weakens the patient by destroying bone-marrow cells. Since bone marrow is responsible for producing blood cells, patients undergoing chemotherapy commonly suffer blood deficiencies. Now that scientists can perform gene therapy in mice, Cline says, they should theoretically be able to do it in cancer patients. Patients might be given the gene for methotrexate resistance in their bone-marrow cells. Then the drug would kill cancerous tissue without harming healthy tissue or impairing blood production. "If we could increase the resistance of cancer patients to these toxic side effects," Cline says, "we might be able to increase the use of methotrexate two- to fourfold."

Lester Sloan—Newsweek

Cline, Mercola: A hope for cancer therapy

More sophisticated gene therapy, however, depends on resolving some unanswered biological questions. Scientists do not know, for instance, exactly where in the bone-marrow cell the gene ends up. Does it correctly enter the right chromosome, a string of hereditary material that contains genes, or does it simply float around in the cell? Getting it to the right place "presents numerous problems," warns Richard Axel of Columbia University, a pioneer in mammalian-gene transfer. For one thing, a free gene is easy to lose—it might simply leak out of the cell. More serious, a gene outside the chromosomes isn't subject to normal genetic regulation. Without such regulation the gene might run wild, producing a surplus of products as dangerous as a deficit. In addition, even if an inserted healthy gene winds up in the chromosome of the defective gene it is meant to replace, there is no guarantee that it will work.

Brave New World: The UCLA scientists are confident that such technical puzzles will be solved. When they are, two natural targets for gene therapy are sickle-cell anemia and thalassemia, blood diseases that arise from defects in single genes. Since mice have now been shown to accept foreign genes into their marrow cells, human patients with defective blood cells might do the same. Doctors would insert two genes linked together, one to correct the defect and one to confer resistance to a toxic drug. If the defective cell does not accept the genes, it would be killed by the drug. If the cell does accept the genes—thus becoming cured—it would also resist the drug. Since only these healthy cells could survive, the treatment would produce a whole population of normal blood cells. The same technique might eventually be used to insert the gene for insulin in diabetics and the gene for growth hormone in patients with dwarfism. When scientists take these steps, the brave new world of gene therapy will have arrived.

SHARON BEGLEY with bureau reports

TRANSITION

EXPECTING: Julie Nixon Eisenhower, 31, and **David Eisenhower,** 32, their second child and former President Nixon's third grandchild; next October. The couple now have a 1-year-old daughter, Jennie.

DIED: Kay Medford, 59, comic actress of stage and films, of cancer; in New York City, April 10. A critic panning a Broadway play took pains to compliment Medford as "a comedienne with the funniest drooping mien since Zasu Pitts," and indeed, the gravelly-voiced actress often received favorable notice even when the shows failed. Among her memorable roles in hits: playing Barbra Streisand's mother in "Funny Girl" both on Broadway and in the film (the movie role won her an Oscar nomination) and Dick Van Dyke's mother in the stage version of "Bye Bye Birdie." "Sure, there's plenty of action in these mama roles," she once said. "But I've never even been a bride."

John Collier, 78, British-born writer, of a stroke; in Pacific Palisades, Calif., April 6. A master of the macabre, Collier was best known for his prize-winning collection of short stories, "Fancies and Goodnights." He also wrote a number of fanciful novels, major screenplays including "I Am a Camera" and "The African Queen" (for which

Malcolm Braly, 54, ex-convict and acclaimed novelist, from injuries suffered in an automobile accident; in Baltimore, April 7. Braly served a total of eighteen years in prison, mostly on charges of robbery. He took a hard, unsentimental view of prison life and wrote of the experience with gritty detail, but without a tone of self-indulgence. His book "On the Yard" was hailed by Kurt Vonnegut as "the great American prison novel."

UNITED STATES DEPARTMENT OF COMMERCE
Patent and Trademark Office
Address : COMMISSIONER OF PATENTS AND TRADEMARKS
Washington, D.C. 20231

SERIAL NUMBER	FILING DATE	FIRST NAMED APPLICANT	ATTORNEY DOCKET NO.
06/124,513	02/25/80	AXEL R	

JOHN P. WHITE
COOPER, DUNHAM, CLARK, GRIFFIN & MORAN
30 ROCKEFELLER PLAZA
NEW YORK, NY 10020

EXAMINER	
KEPPLINGER, E	
ART UNIT	**PAPER NUMBER**
172	9

DATE MAILED: 02/08/82

This is a communication from the examiner in charge of your application.

COMMISSIONER OF PATENTS AND TRADEMARKS

☐ This application has been examined. ☑ Responsive to communication filed on 11/9/81 ☐ This action is made final.

A shortened statutory period for response to this action is set to expire 3 month(s), ~~_____~~ days from the date of this letter.
Failure to respond within the period for response will cause the application to become abandoned. 35 U.S.C. 133

Part I THE FOLLOWING ATTACHMENT(S) ARE PART OF THIS ACTION:

1. ☑ Notice of References Cited by Examiner, PTO-892
2. ☐ Notice of Informal Patent Drawing, PTO-948
3. ☐ Notice of References Cited by Applicant, PTO-1449
4. ☐ Notice of Informal Patent Application, Form PTO-152
5. ☐ _____

Part II SUMMARY OF ACTION

1. ☑ Claims 1-9, 12-21, 23-54, 57-90, 93+116, 118, 119, 124, 125 are pending in the application.
   Of the above, claims 43, 44, 79, 80, 111, 112, 124, 125 are withdrawn from consideration.
2. ☐ Claims _____ have been cancelled.
3. ☐ Claims _____ are allowed.
4. ☑ Claims 1-9, 12-21, 23-42, 45-54, 57-78, 81-90, 93-110, 113-116, 118 and 119 are rejected.
5. ☐ Claims _____ are objected to.
6. ☐ Claims _____ are subject to restriction or election requirement.
7. ☐ The formal drawings filed on _____ are acceptable.
8. ☐ The drawing correction request filed on _____ has been ☐ approved. ☐ disapproved.
9. ☐ Acknowledgment is made of the claim for priority under 35 U.S.C. 119. The certified copy has
   ☐ been received. ☐ not been received. ☐ been filed in parent application, serial no. _____,
   filed on _____.
10. ☐ Since this application appears to be in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under Ex parte Quayle, 1935 C.D. 11; 453 O.G. 213.
11. ☐ Other

PTOL-326 (rev. 7-79)

GENE-CEN 083160

EXAMINER'S ACTION
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119 are rejected under 35 USC 112, first paragraph, as based on a specification which is non-enabling for reasons of record. Applicants urge that although recovery of β-globin is not shown, one skilled in the art would expect production of the protein based the formation of the MRNA. That merely shows transcription, not translation and translation is not assured. For example, a function ribosome binding site may not be present. Production of the protein is not shown. Applicants further urge that thymidine kinase is shown to be produced and recovered. Thymidine kinase, however, is the protein produced by the DNA, recovery of the protein from DNA I. Thus, it is not shown.

Applicants point to two references, Lai et al and Graf et al, as evidence that others used applicants' method to produce protein. Those references show the DNA mediated co-transformation of cells using the method of Wigler et al. It is not clear, however, that the use of different cells as transformants could result in protein formation in the references but not in the specification. Moreover, it is not clear that the eucaryotic cells perform the extra steps such as glycosylation on the proteins to form interferon, for example.

13. Claims 28-30, 67 and 70 are rejected under 35 USC 102 as fully met by either Wacker reference. Applicants urge that the instant claims are not directed to fused cells. The claims merely recite a eucaryotic cell which contains foreign DNA which codes for protein and is not selectable. That is shown by Wacker.

14. Claims 1-9, 12-21, 23-42, 45-54, 57-78, 81-90, 93-110, 113-116, 118 and 119 are rejected under 35 USC 103 as unpatentable over Lai et al who teach the cotransformation of mouse cells with unliked ovalbumin gene

incorporated into the chromosomal DNA with recovery of the protein formed. The use of the genes for other proteins and other selectable characteristics would be obvious. Purification and use of calcium phosphate are conventional in genetic engineering and would be obvious. This insertion of multiple copies of the foreign DNA is shown and to use multiple copies of the selectable gene would be obvious. Applicants present a declaration under 37 CFR 1.131 to remove Ladi et al as a reference. The publications used as evidence of reduction to practice show in all cases that more than the instant three inventors reduced the invention to practice. They do not show that the present inventive entity made the invention and thus the declaration is insufficient. Disclaiming affidavits from all non-inventors are necessary. Moreover, none of the articles show the production of protein of the DNA I so that aspect of the invention does not have basis in the article, and thus may have an effective date only of the filing date. In regard to the Pellicer et al article (PNAS, 77(4, 2098(1980)), it is not clear how applicants arrived at the date of December 31, 1979. January 10, 1980 is the only verified date and it is not clear when in January Ladi et al was published.

14. Claims 25-30, 65-68, 70, 81--97, 100-110 are rejected under 35 USC 103 as unpatentable over Mantei et al for reasons of record. Applicants urge that the use of linked DNA as in Mantei et al is patentably distinct from the instant claims. The cells after transformation, however, do not appear to be different regardless of whether the DNA used was linked or unlinked. Moreover, claims 81+ recite the use of linked DNA. Applicants declaration under 37 CFR 1.131 is insufficient, as set forth above, because disclaiming affidavits from the co-authors are necessary.

[illegible], 113, 114 and 118 are rejected under 35 USC 102 as anticipated by Wigler et al (w), Wigler et al (v) or Wigler et al (x). Applicants declaration under 37 CFR 1.131 is insufficient. Disclaiming affidavits fron all co-authors are required to remove the references. See MPEP 715.01(c).

16. Claims 3-8, 13, 15, 24, 30-42, 47-52, 57, 59, 69, 71-78, 115, 116 and 119 are rejected under 35 USC 103 as unpatentable over Wigler et al (w), (v) or (x) for reasons of record. Disclaiming affidavits from all co-authors are required. See MPEP 715.01(c).

17. Claims 1, 2, 9, 12, 14, 16-18, 23, 25-29, 45, 46, 53, 54, 58, 60, 61, 64-68, 70, 113, 114 and 118 are rejected under 35 USC 102 as fully met by Wold et al. Disclaiming affidavits from the co-authors who are not inventors are required. See MPEP 715.01(c).

18. Claims 3-8, 13, 15, 19-21, 24, 30-42, 47-52, 57, 59, 62, 63, 69, 71-78, 115, 116 and 119 are rejected under 35 USC 103 as unpatentable over Wold et al for reasons of record. In order to remove the reference, disclaiming affidavits from all co-authors who are not inventors are required. See MPEP 715.01(c).

19. Wahl et al, newly cited, show gene amplification by increasing concentrations of a selective agent. Miller et al show calcium phosphate treatment increases transformation efficiency.

20. The specification is objected to as non-enabling because it would not enable one to practice the invention without undue experimentation. No details or examples are given to show how to perform the invention

EKEPPLINGER:hm
703/557-3685
1/26/82

Esther M. Keppplinger

ESTHER M. KEPPLINGER
PRIMARY EXAMINER

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GENE-CEN 083163

TO SEPA... ...RD BOTTOM EDGES, SNAP - A... CARD CARBON

FORM PTO-892 (REV. 3-78) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

NOTICE OF REFERENCES CITED

SERIAL NO.	GROUP ART UNIT	ATTACHMENT TO PAPER NUMBER
124513	172	9

APPLICANT(S): Axel et al.

1/22

U.S. PATENT DOCUMENTS

*		DOCUMENT NO.	DATE	NAME	CLASS	SUB-CLASS	FILING DATE IF APPROPRIATE
	A						
	B						
	C						
	D						
	E						
	F						
	G						
	H						
	I						
	J						
	K						

FOREIGN PATENT DOCUMENTS

*		DOCUMENT NO.	DATE	COUNTRY	NAME	CLASS	SUB-CLASS	PERTINENT SHTS. DWG	PERTINENT PP. SPEC.
	L								
	M								
	N								
	O								
	P								
	Q								

OTHER REFERENCES (Including Author, Title, Date, Pertinent Pages, Etc.)

	R	Wahl et al, J. Biol. Chem., 254(17), 8679-8689 (Sept. 10, 1979).
✓	S	Anderson et al, Scientific American, 245(1), July 1981, 106-121.
✓	T	Fox, C & E News, 35 36, 42 Sept. 29, 1980.
✓		Marx, Science, 208, 386-387, April 1980.
✓	U	Bagley, Newsweek, 80, April 21, 1980.
		Miller et al, PNAS, 75(7), 3346-3350 (1978). ✓

EXAMINER	DATE
E. M. Kepplinger	1/22/82

* A copy of this reference is not being furnished with this office action. (See Manual of Patent Examining Procedure, section 707.05 (a).)

GENE-CEN 083164

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UNITED STATES DEPARTMENT OF COMMERCE
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SERIAL NUMBER	FILING DATE	FIRST NAMED APPLICANT	ATTORNEY DOCKET NO.
124,513	2/25/80	AXEL	

EXAMINER	
E. Kepplinger	
ART UNIT	PAPER NUMBER
172	10

DATE MAILED:

EXAMINER INTERVIEW SUMMARY RECORD

All participants (applicant, applicant's representative, PTO personnel):

(1) John White (3) _____

(2) _____ (4) _____

Date of interview 2/23/82 and 2/27/82

Type: ☑ Telephonic ☐ Personal (copy is given to ☐ applicant ☐ applicant's representative).

Exhibit shown or demonstration conducted: ☐ Yes ☑ No. If yes, brief description: _____

Agreement ☐ was reached with respect to some or all of the claims in question. ☑ was not reached.

Claims discussed: all

Identification of prior art discussed: Lai, Mantei and [redacted] Wigler (W), (V), and (X), and Wold

Description of the general nature of what was agreed to if an agreement was reached, or any other comments: I indicated that since the references co-authored by applicants and additional authors provide evidence that others contributed to the invention, more evidence is required to eliminate those additional people. Evidence of conception by applicants and description of what each person did or disclaiming affidavits from the coauthors of W, V, X and Wold who are not inventors would obviate the

(A fuller description, if necessary, and a copy of the amendments, if available, which the examiner agreed would render the claims allowable must be attached. Also, where no copy of the amendments which would render the claims allowable is available, a summary thereof must be attached.)

Unless the paragraphs below have been checked to indicate to the contrary, A FORMAL WRITTEN RESPONSE TO THE LAST OFFICE ACTION IS NOT WAIVED AND MUST INCLUDE THE SUBSTANCE OF THE INTERVIEW (e.g., items 1–7 on the reverse side of this form). If a response to the last Office action has already been filed, then applicant is given one month from this interview date to provide a statement of the substance of the interview.

☐ It is not necessary for applicant to provide a separate reocrd of the substance of the interview.

☐ Since the examiner's interview summary above (including any attachments) reflects a complete response to each of the objections, rejections and requirements that may be present in the last Office action, and since the claims are now allowable, this completed form is considered to fulfill the response requirements of the last Office action.

GENE-CEN 083165

81-3679 PTOL-413 (rev. 1-81) 191 Examiner's Signature

Page 1

D17668-A JPW

#11
4/30/82

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Richard Axel, et al.

Serial No.: 124,513 Examiner: Esther Kepplinger

Filed : February 25, 1980 Group Art Unit: 172

For : PROCESSES FOR INSERTING DNA INTO EUCARYOTIC CELLS AND FOR PRODUCING PROTEINACEOUS MATERIALS

30 Rockefeller Plaza
New York, New York 10112

28 April 1982

Honorable Commissioner of Patents
and Trademarks
Washington, D.C. 20231

Sir:

FIRST REQUEST FOR A ONE MONTH EXTENSION OF TIME

It is respectfully requested that the period for filing a response to the February 8, 1982 Official Action issued in connection with the subject application, now set to expire May 8, 1982, be extended one month so as to expire June 8, 1982.

This is the first request for an extension of time for filing a response to the February 8, 1982 Official Action; it is not anticipated that another request will be submitted.

After the February 8, 1982 Official Action was received, a letter reporting on the Action, together with copies of the Official Action, was mailed to applicants. Subsequently, applicants' undersigned attorney conferred with applicants by telephone and in person. Moreover, on April 20, 23 and 27, applicants' undersigned attorney conferred with the Examiner handling the application in an effort to clarify certain issues. In view of the numerous issues which exist, applicants and applicants' attorney require additional time to confer so as to be able to prepare a complete response to the Official Action.

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GENE-CEN 083166

It is therefore requested that a one month extension of the deadline for filing a response, from May 8, 1982 to June 8, 1982, be favorably considered and granted.

Respectfully submitted,

John P White

John P. White
Reg. No. 28,678
Attorney for Applicants

(212) 977-9550

APPROVED

APR 29 1982

RUTH W. LYLES
S.P.A.

D17668-A JPW

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Richard Axel, et al.

Serial No.: 124,513 Examiner: Esther M. Kepplinger

Filed : February 25, 1980 Group Art Unit: 172

For : PROCESSES FOR INSERTING DNA INTO EUCARYOTIC CELLS AND FOR PRODUCING PROTEINACEOUS MATERIALS

30 Rockefeller Plaza
New York, New York 10112

7 June 1982

Honorable Commissioner of Patents
and Trademarks
Washington, D.C. 20231

SIR:

AMENDMENT

This Amendment is submitted in response to the February 8, 1982 Official Action issued in connection with the above-identified application. A response to that Official Action is presently due no later than June 8, 1982. Please amend the subject application as follows:

IN THE SPECIFICATION:

On page 1, after the title, insert the following:

~~The~~ invention described herein was made in the course of work under grants numbers CA-23767 and CA-76346 from the National Institutes of Health, Department of Health and Human Services.~~

IN THE CLAIMS:

Cancel claims 28-30, 38, 67-70, 74, 115, 116 and 119.

GENE-CEN 083168

Further amend claim 1 as follows:

~~1~~(twice amended). A process for inserting foreign DNA I into a suitable eucaryotic cell which comprises cotransforming said eucaryotic cell with said foreign DNA I and with unlinked foreign DNA II which codes for a selectable phenotype not expressed by said eucaryotic cell, said cotransformation being carried out under suitable [in a suitable medium and in the presence of selective] conditions permitting survival or identification of eucaryotic cells which have acquired said selectable phenotype, said foreign DNA I being incorporated into the chromosomal DNA of said eucaryotic cell.

Further amend claim 23 as follows:

~~23~~ 28 (twice amended). A process for inserting purified foreign DNA I coding for proteinaceous material which is not associated with a selectable phenotype into a suitable eucaryotic cell which comprises cotransforming said eucaryotic cell with said foreign DNA I [coding for proteinaceous material which is not associated with a selectable phenotype] and with [physically] unlinked foreign DNA II coding for proteinaceous material which is associated with a selectable phenotype, said cotransformation being carried out under suitable [in the presence of selective] conditions permitting survival or identification of eucaryotic cells which have acquired said selectable phenotype, said foreign DNA I being incorporated into the chromosomal DNA of said eucaryotic cell.

Amend claim 35 as follows:

~~35~~ 22 (amended). A process for producing a foreign proteinaceous material which comprises cotransforming a eucaryotic cell [with foreign DNA I coding for said proteinaceous

material] in accordance with the process of Claim 1, culturing or cloning said cotransformed eucaryotic cell under suitable [selective] conditions to [produce] yield a multiplicity of eucaryotic cells producing said foreign proteinaceous material [derived therefrom] and recovering said proteinaceous material from [the] said eucaryotic cells [so produced].

Amend claim 39 as follows:

39(amended). A process for producing proteinaceous material which is not associated with a selectable phenotype which comprises cotransforming a eucaryotic cell with foreign DNA I coding for said proteinaceous material in accordance with the process of claim 23, culturing or cloning said cotransformed eucaryotic cell under suitable [in the presence of said selective] conditions to produce a multiplicity of eucaryotic cells derived therefrom, and recovering said proteinaceous material from the eucaryotic cells so produced.

Amend claim 45 as follows:

~~45~~ 31(amended). A process for inserting a multiplicity of foreign DNA I molecules corresponding to multiple copies of a gene coding for a proteinaceous material into a suitable eucaryotic cell which comprises cotransforming said eucaryotic cell with said multiplicity of foreign DNA I molecules and with a multiplicity of unlinked foreign DNA II molecules coding for a selectable phenotype not expressed by said eucaryotic cell, said cotransformation being carried out [in a suitable medium and in the presence of an agent] under suitable conditions permitting survival or identification of eucaryotic cells which have acquired said multiplicity of genes coding for said selectable phenotype.

Amend claim 71 as follows:

~~71~~ 51 ~~(amended)~~. A process for producing a foreign proteinaceous material which comprises cotransforming a eucaryotic cell [with a multiplicity of foreign DNA I molecules coding for said proteinaceous material] in accordance with the process of claim ~~45~~ 31, maintaining said cotransformed eucaryotic cell under suitable conditons to [enable said foreign DNA I to be transcribed to form complementary RNA and said complementary RNA so formed to be translated to] produce said foreign proteinaceous material, and recovering said proteinaceous material so produced.

B7

Amend claim 75 as follows:

~~75~~ (amended). A process for producing proteinaceous material which comprises cotransforming a eucaryotic cell with a multiplicity of foreign DNA I molecules coding for said proteinaceous material in accordance with the process of claim 45, culturing or cloning said cotransformed eucaryotic cell under suitable conditions [in the presence of said agent] permitting survival or identification of eucaryotic cells which have acquired said multiplicity of genes coding for said selectable phenotype to produce a multiplicity of eucaryotic cells derived therefrom, and recovering said proteinaceous material from the eucaryotic cells so produced.

B8

Amend claim 81 as follows:

Change the last four words from "in a suitable medium" to under suitable conditions.

Further amend claim 113 as follows:

~~113~~ 27 (twice amended). A process for cotransforming a suitable eucaryotic cell which comprises transforming under

B9

~~74~~ 114

suitable conditions said eucaryotic cell with foreign DNA I and with foreign DNA II, said DNA I and DNA II being unlinked and said DNA II coding for a selectable phenotype not expressed by said eucaryotic cell prior to cotransformation.

REMARKS

This Amendment and the accompanying SUPPLEMENTAL DECLARATION UNDER 37 C.F.R. § 1.131 annexed hereto as Exhibits A-C are submitted in response to the February 8, 1982 Official Action issued in connection with the subject application.

Before proceeding, applicants' undersigned attorney wishes to thank the Examiner for the courtesy extended during a telephone interview in which the Examiner clarified the bases for several of her rejections. Applicants herein attempt to respond to the specific comments and suggestions made by the Examiner and believe that this Amendment, together with the accompanying SUPPLEMENTAL DECLARATION, should place the application in condition for allowance.

Applicants now turn to the specific grounds for rejection set forth in the February 8 Official Action. In paragraph 12 of the Official Action, the Examiner rejected claims 31-42, 71-78, 103-110, 115, 116 and 119 under 35 U.S.C. §112, first paragraph, as allegedly based upon a non-enabling specification because only production of thymidine kinase, not protein encoded by DNA I, is shown.

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GENE-CEN 083172

With respect to claims 35-38 and 71-74, applicants have cancelled claims 38 and 74 and have amended claims 35 and 71. As so amended, claims 35 and 71 and the claims dependent thereon, 36-37 and 72-73, respectively, do not require that the protein which is produced be encoded by DNA I. Thus, claims 35-37 and 71-73 as amended cover production and recovery of thymidine kinase which, as the Examiner acknowledged in the Official Action, is disclosed.

Applicants therefore request that the Examiner reconsider the rejection in so far as it concerns amended claims 35-37 and 71-73.

With respect to claims 31-34, 39-42, 75-78 and 103-110, applicants direct the Examiner's attention to the Lai, et al and Graf, et al references, U and WW, respectively. These references show that as of applicants' 25 February 1980 filing date, persons skilled in the art knew that protein encoded by DNA I could be produced using applicants' claimed process and had done so.

Thus, in reference U, Lai et al report "the transformation of mouse LMTK$^-$ cells with the chromosomal ovalbumin gene by using this DNA-mediated gene transfer system", referring to Wigler, et al., Cell, Vol. 16, pp 777-785 (1979), reference V herein, corresponding to the "First Series of Experiments" on pages 22-35 of the specification. The Examiner's suggestion that Lai, et al used different cells is not supported by the facts. Lai, et al identify one of two strains of mouse LMTK$^-$ cells used as being generously provided by R. Axel, Columbia University (see pages 244 and 248).

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Similarly, Graf, et al report that they used the transformation system of Wigler, et al to produce the protein hypoxanthine phosphoribosyltransferase as confirmed by enzymatic activity in extracts of transformed cells. Graf, et al's mouse cell line LMTK$^-$ was provided by S. Silverstein (see page 1032). Moreover, Graf, et al in acknowledgements on page 1043 thank Michael Wigler and Angel Pellicer for their help in the initial tk transformation experiments.

In view of the foregoing, applicants maintain that their specification would and did enable persons skilled in the art to produce and recover protein encoded by DNA I. Applicants therefore maintain that their disclosure is enabling and request that the Examiner reconsider the rejection under 35 U.S.C. § 112, first paragraph.

In this regard, applicants direct the Examiner's attention to the recent decision of the Court of Customs and Patent Appeals in In re Strahilevitz, 212 USPQ 561 (1982). A copy of this decision in which the pertinent portions are highlighted is annexed hereto as Exhibit D.

As clearly indicated in this decision, the Patent Office has the burden of proof in calling into question the enablement of a disclosure and must advance acceptable reasoning inconsistent with enablement. As further indicated therein, examples are not required to satisfy section 112, first paragraph. Finally, as stated therein, the dispositive issue is whether the disclosure, considering the level of skill in the art as of the date of the application, would have enabled a person of such skill to make and use the invention without undue

-7- 179

experimentation. As stated on page 364 of this decision, first full paragraph, an applicant may properly rely on literature citations to establish both the level of skill in the art and the fact that the techniques necessary to practice the invention are known in the art. In view of the prior publication dates of the Lai, et al and Graf, et al references, applicants maintain that this dispositive issue must clearly be resolved in their favor since others skilled in the art had already made and used their invention without undue experimentation as of applicants' February 25, 1980 filing date.

With respect to claims 115, 116 and 119, applicants maintain that the Examiner's rejection of these claims on the ground that it is allegedly not clear that eucaryotic cells perform the extra steps such as glycosylation on the proteins to form interferon, for example, has been rendered moot. These claims have been cancelled in order to advance prosecution of the broader claims pending in this application. However, such cancellation is made without prejudice to applicants' right to present these claims at a future time in any continuation or other related application which may be filed.

In paragraph 13 of the Official Action, the Examiner rejected claims 28-30, 67 and 70 under 35 U.S.C. § 102. In response, applicants have cancelled claims 28-30 and 67-70. The cancellation of additional claims 68-69 is deemed necessary for consistency.

The Examiner, in paragraph 14 of the Official Action, rejected claims 1-9, 12-21, 23-42, 45-54, 57-78, 81-90, 93-110, 113-116, 118 and 119 under U.S.C. § 103 on the ground that

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the invention claimed allegedly would have been obvious when made to one skilled in the art given the teachings of Lai, et al. The Examiner referred to applicants' previously submitted Declaration Under 37 C.F.R. § 1.131, indicating that it is insufficient because the publications used as evidence of reduction to practice name coauthors in addition to applicants, and requiring that disclaiming affidavits from non-inventor, coauthors be submitted.

Based upon the telephone interview which was held between applicants' undersigned attorney and the Examiner, it is believed that agreement was reached that a sufficient declaration under 37 C.F.R. § 1.131 would be a basis for removing Lai, et al. as a reference since disclaimers are a permissible, but not mandatory, alternative to Rule 131 affidavits or declarations. Applicants therefore submit the accompanying SUPPLEMENTAL DECLARATION UNDER 37 C.F.R. § 1.131, annexed hereto as Exhibits A-C. This SUPPLEMENTAL DECLARATION explains the role of various non-inventor, coauthors and is sufficient to obviate any question concerning inventorship. Accordingly, applicants request that the Examiner remove Lai, et al as a reference and reconsider the rejection under 35 U.S.C. § 103.

In paragraph 13, the Examiner further noted that the articles relied upon to show production of protein from DNA I do not do so. Applicants have previously pointed out that the articles show production of thymidine kinase and have amended claims 35-37 and 71-73 to cover that embodiment of their invention which concerns production of protein generally. As to these claims, applicants again request reconsideration.

-8-
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As to those claims specifically directed to production of protein from DNA I, applicants' accompanying SUPPLEMENTAL DECLARATION, read in conjunction with the record in this case, shows that applicants conceived their invention prior to the January 1980 publication date of the Lai, et al article. Applicants exercised diligence from at least as early as December 1979 until the February 25, 1980 filing date of the subject application. Accordingly, Lai, et al should also be removed as a reference as to claims directed to production of protein from DNA I.

In regard to Pellicer, et al., PNAS 77:2098-2102, April 1980, the Examiner indicated that it was not clear how Applicants arrived at the date of December 31, 1979 since January 10, 1980 was the only verified date. In response, applicants point out that January 10, 1980 is the date the manuscript is acknowledged by the journal as having been communicated. Since it obviously required at least 10 days to compile the experimental results and prepare the manscript, it is reasonable that the experiments were completed at least as early as December 31, 1979 and applicants have so declared pursuant to 37 C.F.R. § 1.131. The significance of these dates or of the precise date in January when Lai, et al was published is not apparent to applicants since the protein whose production is shown in Pellicer, et al is thymidine kinase, production of which was demonstrated in earlier experiments involving selection of tk^+ cotransformants.

In view of the applicants' arguments and the accompanying SUPPLEMENTAL DECLARATION, they respectfully request that the Examiner remove Lai, et al as a reference and reconsider the rejection of claims over Lai, et al.

In paragraph 14, the Examiner rejected claims 25-30, 65-68, 70, 81-97 and 100-110 under 35 U.S.C. § 103 over Mantei, et al. Once again, the Examiner required disclaiming affidavits from noninventor, coauthors. Applicants request that, in view of their accompanying SUPPLEMENTAL DECLARATION, the Examiner remove Mantei, et al as a reference and reconsider the rejection of claims over Mantei, et al.

In paragraphs 15, 16, 17 and 18, various subgroupings of claims were rejected under 35 U.S.C. § 102 or § 103 on the basis of references V, W, X or Y, each of which names applicants as coauthors.

In view of the accompanying SUPPLEMENTAL DECLARATION setting forth details concerning the roles of noninventor, coauthors and again pointing out that Y concerns transfomation, not cotransformation, applicants request that the Examiner remove V, W, X and Y as references and reconsider the rejections based upon these references.

Applicants have noted the additional references cited, but not applied, by the Examiner, including the four references which were called to the Examiner's attention in applicants' communication filed on November 11, 1981.

Applicants have also noted the Examiner's objection to the specification. For reasons set forth hereinabove and in the In re Strahilevitz case annexed hereto as Exhibit D, applicants again request that the Examiner reconsider.

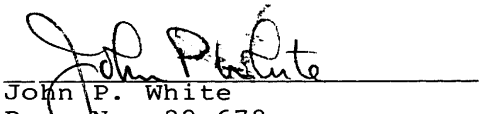
Finally, applicants, in order to more clearly, accurately and patentably define their invention, have made a number of minor amendments in various claims. These amendments do not involve new matter, do not require a new search, and are fully supported by the specification. Also, in order to clarify the interest of the U.S. Government in the application, an amendment for this purpose has been made at the beginning of the specification.

In conclusion, applicants maintain that the claims now pending in this application accurately and patenably define their invention and are patentably distinguishable over the prior art, particularly in view of the accompanying SUPPLEMENTAL DECLARATION UNDER 37 C.F.R. § 1.131. Accordingly, applicants request that the Examiner reconsider the various grounds for rejection and objection set forth in the outstanding February 8, 1982 Official Action and earnestly solicit allowance of the claims now pending.

If a telephone interview would be of assistance in advancing prosecution of the application, applicants' undersigned attorney invites the Examiner to telephone him at the number provided.

No fee is deemed necessary in connection with this Amendment. If a fee is required, authorization is hereby given to charge the amount of any such fee to Deposit Account No. 03-3125.

Respectfully submitted,



John P. White
Reg. No. 28,678
Attorney for Applicants
212-977-9550

-22-

GENE-CEN 083179

D17668-A JPW

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Richard Axel, et al.

Serial No.: 124,513 Examiner: Esther M. Kepplinger

Filed : February 25, 1980 Group Art Unit: 172

For : Processes for Inserting DNA Into Eucaryotic Cells and For Producing Proteinaceous Materials

Honorable Commissioner of Patents
and Trademarks
Washington, D.C. 20231

SIR:

SUPPLEMENTAL DECLARATION UNDER 37 C.F.R. § 1.131

noted
Enk
8/20/82

We, RICHARD AXEL, MICHAEL H. WIGLER and SAUL J. SILVERSTEIN, declare as follows:

1. We are the RICHARD AXEL, MICHAEL H. WIGLER and SAUL J. SILVERSTEIN who are named as applicants on the above-identified patent application.

2. We previously submitted a DECLARATION UNDER 37 C.F.R. § 1.131 signed by us on November 2, November 1 and November 3, 1981, respectively.

3. We have read the Official Action issued in connection with the subject application on February 8, 1982. We understand from the Official Action and from our attorney, Mr. John P. White, that the Patent Office Examiner handling our application requires additional information concerning the respective roles of persons named as coauthors, including ourselves, on certain cited references as follows:

Richard Axel
Serial No. 124,513
Group Art Unit 172

EXHIBIT A

8/24

GENE-CEN 083180

	Reference	Coauthors
V:	Cell, Vol. 16, pp 777-785, April 1979	Wigler, Michael Sweet, Raymond Sim, Gek Kee Wold, Barbara Pellicer, Angel Lacy, Elizabeth Maniatis, Tom Silverstein, Saul Axel, Richard
W:	Eucaryotic Gene Regulation, Proc. ICN-UCLA Symposium, R. Axel and T. Maniatis, editors, Academic Press, pp 457-475, December 1979	Wigler, Michael Pellicer, Angel Silverstein, Saul Axel, Richard Urlaub, Gail Chasin, Lawrence
X:	PNAS, Vol. 76, pp 1373-1376, March 1979	Wigler, Michael Pellicer, Angel Silverstein, Saul Axel, Richard Urlaub, Gail Chasin, Lawrence
Y:	PNAS, Vol. 76, pp 5684-5688, November 1979	Wold, Barbara Wigler, Michael Lacy, Elizabeth Maniatis, Tom Silverstein, Saul Axel, Richard

2205

4. The invention described and claimed in the subject application was conceived solely by the three of us at least as early as January 8, 1979.

5. Subsequent to and in accordance with our conception, one of us, MICHAEL H. WIGLER, personally and solely conducted the first experiments reducing the invention to practice. These experiments which were carried out in the United States involved cotransformation of mouse Ltk$^-$ cells with ΦX174 DNA and with the tk gene. These experiments are described in reference V at pages 777-781. The manuscript which was eventually published as reference V was received by Cell initially on January 8, 1979 and in revised form on January 29, 1979 (see page 785 of reference V). Therefore, it is clear that an actual reduction to practice occurred at least as early as January 8, 1979.

6. After the ΦX cotransformation experiments were performed, MR. RAYMOND SWEET, a postdoctoral student who had experience with the technique of blot hybridization, was provided samples of cotransformed cells and asked to confirm that the ΦX DNA was present in them. MR. SWEET did so. He later did confirmatory blot hybridizations on samples cotransformed with other DNAs. On the basis of these blot hybridizations, MR. SWEET was named as a coauthor on references V and W.

7. In order to confirm the generality of the cotransformation discovery, two other postdoctoral students, MS. GEK KEE SIM and MS. BARBARA WOLD, working in the laboratory and under direction of one of us, RICHARD AXEL, successfully cotransformed mouse cells with pBR322 DNA and with the rabbit β major globin

gene, respectively. Each used the same method as that used in the ΦX cotransformation. MS. SIM and MS. WOLD were then named as coauthors on references V and W.

8. During the course of the experiments subsequent to the ΦX experiments, MR. ANGEL PELLICER, another postdoctoral student in the laboratory of one of us, RICHARD AXEL, provided the persons who were actually conducting the experiments with practical advice concerning certain of the laboratory techniques employed. MR. PELLICER was named as a coauthor on references V, W and X.

9. In order to carry out experiments with the globin gene, we obtained the gene from MS. ELIZABETH LACY and MR. TOM MANIATIS. MR. MANIATIS was a professor at the California Institute of Technology and MS. LACY was working in his laboratory. Because MS. LACY and MR. MANIATIS provided the globin gene, they were named as coauthors on references V, W and Y.

10. In order to confirm the generality of the earlier work involving transformation, not cotransformation, experiments were carried out using the aprt gene as the selectable marker. Assays to confirm the presence of the enzyme aprt in transformed cells were carried out in the laboratory of MR. LAWRENCE CHASIN by MS. GAIL URLAUB. MR. CHASIN and MS. URLAUB were then named as coauthors on reference X. However, as stated in paragraph 10 of our previous DECLARATION, these experiments are not described in the subject application and do not relate to cotransformation.

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11. With respect to the four references cited by the Examiner, V, W, X and Y, we, RICHARD AXEL, MICHAEL H. WIGLER, and SAUL J. SILVERSTEIN, are the only persons named as coauthors on all the references.

12. In summary, we alone conceived the invention described and claimed in the subject application at least as early as January 8, 1979. Subsequently, one of us, MICHAEL H. WIGLER, successfully cotransformed eucaryotic cells in the United States. We then actually showed that the cotransformed cells produced protein (thymidine kinase). Others [Mantei, et al. (reference T) and Lai, et al. (reference U)] actually showed that cells cotransformed by our method produced protein encoded by the cotransforming DNA not associated with the selectable marker; and we constructively did so by filing the subject patent applicaton.

We declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that willful false statements may jeopardize the validity of the application or any patent issuing thereon.

6/1/82
Date

Richard Axel
RICHARD AXEL

Date

MICHAEL H. WIGLER

Date

SAUL J. SILVERSTEIN

GENE-CEN 083184

D17668-A JPW

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Richard Axel, et al.

Serial No.: 124,513 Examiner: Esther M. Kepplinger

Filed : February 25, 1980 Group Art Unit: 172

For : Processes for Inserting DNA Into Eucaryotic Cells and For Producing Proteinaceous Materials

Honorable Commissioner of Patents
and Trademarks
Washington, D.C. 20231

noted
Emk
8/20/82

SIR:

SUPPLEMENTAL DECLARATION UNDER 37 C.F.R. § 1.131

We, RICHARD AXEL, MICHAEL H. WIGLER and SAUL J. SILVERSTEIN, declare as follows:

1. We are the RICHARD AXEL, MICHAEL H. WIGLER and SAUL J. SILVERSTEIN who are named as applicants on the above-identified patent application.

2. We previously submitted a DECLARATION UNDER 37 C.F.R. § 1.131 signed by us on November 2, November 1 and November 3, 1981, respectively.

3. We have read the Official Action issued in connection with the subject application on February 8, 1982. We understand from the Official Action and from our attorney, Mr. John P. White, that the Patent Office Examiner handling our application requires additional information concerning the respective roles of persons named as coauthors, including ourselves, on certain cited references as follows:

Richard Axel
Serial No. 124,513
Group Art Unit 172

EXHIBIT B

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GENE-CEN 083185

	Reference	Coauthors
V:	Cell, Vol. 16, pp 777-785, April 1979	Wigler, Michael Sweet, Raymond Sim, Gek Kee Wold, Barbara Pellicer, Angel Lacy, Elizabeth Maniatis, Tom Silverstein, Saul Axel, Richard
W:	Eucaryotic Gene Regulation, Proc. ICN-UCLA Symposium, R. Axel and T. Maniatis, editors, Academic Press, pp 457-475, December 1979	Wigler, Michael Pellicer, Angel Silverstein, Saul Axel, Richard Urlaub, Gail Chasin, Lawrence
X:	PNAS, Vol. 76, pp 1373-1376, March 1979	Wigler, Michael Pellicer, Angel Silverstein, Saul Axel, Richard Urlaub, Gail Chasin, Lawrence
Y:	PNAS, Vol. 76, pp 5684-5688, November 1979	Wold, Barbara Wigler, Michael Lacy, Elizabeth Maniatis, Tom Silverstein, Saul Axel, Richard

GENE-CEN 083186

2/10

4. The invention described and claimed in the subject application was conceived solely by the three of us at least as early as January 8, 1979.

5. Subsequent to and in accordance with our conception, one of us, MICHAEL H. WIGLER, personally and solely conducted the first experiments reducing the invention to practice. These experiments which were carried out in the United States involved cotransformation of mouse Ltk$^-$ cells with ΦX174 DNA and with the tk gene. These experiments are described in reference V at pages 777-781. The manuscript which was eventually published as reference V was received by Cell initially on January 8, 1979 and in revised form on January 29, 1979 (see page 785 of reference V). Therefore, it is clear that an actual reduction to practice occurred at least as early as January 8, 1979.

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gene, respectively. Each used the same method as that used in the ΦX cotransformation. MS. SIM and MS. WOLD were then named as coauthors on references V and W.

8. During the course of the experiments subsequent to the ΦX experiments, MR. ANGEL PELLICER, another postdoctoral student in the laboratory of one of us, RICHARD AXEL, provided the persons who were actually conducting the experiments with practical advice concerning certain of the laboratory techniques employed. MR. PELLICER was named as a coauthor on references V, W and X.

9. In order to carry out experiments with the globin gene, we obtained the gene from MS. ELIZABETH LACY and MR. TOM MANIATIS. MR. MANIATIS was a professor at the California Institute of Technology and MS. LACY was working in his laboratory. Because MS. LACY and MR. MANIATIS provided the globin gene, they were named as coauthors on references V, W and Y.

10. In order to confirm the generality of the earlier work involving transformation, not cotransformation, experiments were carried out using the aprt gene as the selectable marker. Assays to confirm the presence of the enzyme aprt in transformed cells were carried out in the laboratory of MR. LAWRENCE CHASIN by MS. GAIL URLAUB. MR. CHASIN and MS. URLAUB were then named as coauthors on reference X. However, as stated in paragraph 10 of our previous DECLARATION, these experiments are not described in the subject application and do not relate to cotransformation.

11. With respect to the four references cited by the Examiner, V, W, X and Y, we, RICHARD AXEL, MICHAEL H. WIGLER, and SAUL J. SILVERSTEIN, are the only persons named as coauthors on all the references.

12. In summary, we alone conceived the invention described and claimed in the subject application at least as early as January 8, 1979. Subsequently, one of us, MICHAEL H. WIGLER, successfully cotransformed eucaryotic cells in the United States. We then actually showed that the cotransformed cells produced protein (thymidine kinase). Others [Mantei, et al. (reference T) and Lai, et al. (reference U)] actually showed that cells cotransformed by our method produced protein encoded by the cotransforming DNA not associated with the selectable marker; and we constructively did so by filing the subject patent applicaton.

We declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date _____ RICHARD AXEL

Date _____ MICHAEL H. WIGLER

06 02 82
Date _____ SAUL J. SILVERSTEIN

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Richard Axel, et al.

Serial No.: 124,513 Examiner: Esther M. Kepplinger

Filed : February 25, 1980 Group Art Unit: 172

For : Processes for Inserting DNA Into Eucaryotic Cells and For Producing Proteinaceous Materials

Honorable Commissioner of Patents
and Trademarks
Washington, D.C. 20231

noted
Emk
8/20/82

SIR:

SUPPLEMENTAL DECLARATION UNDER 37 C.F.R. § 1.131

We, RICHARD AXEL, MICHAEL H. WIGLER and SAUL J. SILVERSTEIN, declare as follows:

1. We are the RICHARD AXEL, MICHAEL H. WIGLER and SAUL J. SILVERSTEIN who are named as applicants on the above-identified patent application.

2. We previously submitted a DECLARATION UNDER 37 C.F.R. § 1.131 signed by us on November 2, November 1 and November 3, 1981, respectively.

3. We have read the Official Action issued in connection with the subject application on February 8, 1982. We understand from the Official Action and from our attorney, Mr. John P. White, that the Patent Office Examiner handling our application requires additional information concerning the respective roles of persons named as coauthors, including ourselves, on certain cited references as follows:

Richard Axel
Serial No. 124,513
Group Art Unit 172

EXHIBIT C

GENE-CEN 083190

	Reference	Coauthors
V:	Cell, Vol. 16, pp 777-785, April 1979	Wigler, Michael Sweet, Raymond Sim, Gek Kee Wold, Barbara Pellicer, Angel Lacy, Elizabeth Maniatis, Tom Silverstein, Saul Axel, Richard
W:	Eucaryotic Gene Regulation, Proc. ICN-UCLA Symposium, R. Axel and T. Maniatis, editors, Academic Press, pp 457-475, December 1979	Wigler, Michael Pellicer, Angel Silverstein, Saul Axel, Richard Urlaub, Gail Chasin, Lawrence
X:	PNAS, Vol. 76, pp 1373-1376, March 1979	Wigler, Michael Pellicer, Angel Silverstein, Saul Axel, Richard Urlaub, Gail Chasin, Lawrence
Y:	PNAS, Vol. 76, pp 5684-5688, November 1979	Wold, Barbara Wigler, Michael Lacy, Elizabeth Maniatis, Tom Silverstein, Saul Axel, Richard

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4. The invention described and claimed in the subject application was conceived solely by the three of us at least as early as January 8, 1979.

5. Subsequent to and in accordance with our conception, one of us, MICHAEL H. WIGLER, personally and solely conducted the first experiments reducing the invention to practice. These experiments which were carried out in the United States involved cotransformation of mouse Ltk$^-$ cells with ΦX174 DNA and with the tk gene. These experiments are described in reference V at pages 777-781. The manuscript which was eventually published as reference V was received by Cell initially on January 8, 1979 and in revised form on January 29, 1979 (see page 785 of reference V). Therefore, it is clear that an actual reduction to practice occurred at least as early as January 8, 1979.

6. After the ΦX cotransformation experiments were performed, MR. RAYMOND SWEET, a postdoctoral student who had experience with the technique of blot hybridization, was provided samples of cotransformed cells and asked to confirm that the ΦX DNA was present in them. MR. SWEET did so. He later did confirmatory blot hybridizations on samples cotransformed with other DNAs. On the basis of these blot hybridizations, MR. SWEET was named as a coauthor on references V and W.

7. In order to confirm the generality of the cotransformation discovery, two other postdoctoral students, MS. GEK KEE SIM and MS. BARBARA WOLD, working in the laboratory and under direction of one of us, RICHARD AXEL, successfully cotransformed mouse cells with pBR322 DNA and with the rabbit β major globin

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gene, respectively. Each used the same method as that used in the ΦX cotransformation. MS. SIM and MS. WOLD were then named as coauthors on references V and W.

8. During the course of the experiments subsequent to the ΦX experiments, MR. ANGEL PELLICER, another postdoctoral student in the laboratory of one of us, RICHARD AXEL, provided the persons who were actually conducting the experiments with practical advice concerning certain of the laboratory techniques employed. MR. PELLICER was named as a coauthor on references V, W and X.

9. In order to carry out experiments with the globin gene, we obtained the gene from MS. ELIZABETH LACY and MR. TOM MANIATIS. MR. MANIATIS was a professor at the California Institute of Technology and MS. LACY was working in his laboratory. Because MS. LACY and MR. MANIATIS provided the globin gene, they were named as coauthors on references V, W and Y.

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2/7

11. With respect to the four references cited by the Examiner, V, W, X and Y, we, RICHARD AXEL, MICHAEL H. WIGLER, and SAUL J. SILVERSTEIN, are the only persons named as coauthors on all the references.

12. In summary, we alone conceived the invention described and claimed in the subject application at least as early as January 8, 1979. Subsequently, one of us, MICHAEL H. WIGLER, successfully cotransformed eucaryotic cells in the United States. We then actually showed that the cotransformed cells produced protein (thymidine kinase). Others [Mantei, et al. (reference T) and Lai, et al. (reference U)] actually showed that cells cotransformed by our method produced protein encoded by the cotransforming DNA not associated with the selectable marker; and we constructively did so by filing the subject patent applicaton.

We declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date	RICHARD AXEL
June 3 1982	M H Wigler
Date	MICHAEL H. WIGLER
Date	SAUL J. SILVERSTEIN

GENE-CEN 083194

File No. D17 ew Client Columb niversity

MAIL ROOM JUN 7 1982 PAT. & TRADE MARK OFF.

IN THE UNITED STATES PATENT OFFICE

RECEIVED JUN 9 1982 GROUP 172

In re application of: Richard Axel et al.

Serial No. 124,513 Examiner: Esther Kepplinger

Filed February 25, 1980 Group Art Unit: 172

For: PROCESSES FOR INSERTING DNA INTO EUCARYOTIC CELLS AND FOR PRODUCING PROTEINACEOUS MATERIALS

The Commissioner of Patents,
Washington, D. C. 20231.

7 June 1982

Sir:

Transmitted herewith is an amendment in the above-identified application.

☐ No additional fee is enclosed because this application was filed prior to October 25, 1965 (effective date of Public Law 89-83).

☒ No additional fee is required.

The fee has been calculated as shown below.

Claims as Amended

(1)	(2) Claims remaining after amendment	(3)	(4) Highest number previously paid for	(5) Present extra	(6) Rate	(7) Additional fee
Total claims.	* 101	Minus	** 125 =	0 ×	$2	= 0.00
Independent claims.	* 8	Minus	11 =	0 ×	10	= 0.00
				Total additional fee for this amendment →		$ 0.00

* If the entry in Column 2 is less than the entry in Column 4, write "0" in Column 5.

** If the "Highest Number Previously Paid For" in this space is less than 10, write "10" in this space.

☐ A check in the amount of $..... is attached.

☐ Charge $ ..... to Deposit Account No. 03-3125. A duplicate copy of this sheet is enclosed.

☒ Please charge any additional fees or credit overpayment to Deposit Account of Cooper, Dunham, Clark, Griffin & Moran No. 03-3125, and notify the undersigned attorney of the amount so charged. A duplicate copy of this sheet is enclosed.

Respectfully,

John P White

Attorney for Applicant
John P. White, Reg. No. 28,678
c/o COOPER, DUNHAM, CLARK, GRIFFIN & MORAN
30 Rockefeller Plaza New York, N. Y. 10020
(212) 977-9550

GENE-CEN 083195

UNITED STATES DEPARTMENT OF COMMERCE
Patent and Trademark Office
Address : COMMISSIONER OF PATENTS AND TRADEMARKS
Washington, D.C. 20231

SERIAL NUMBER	FILING DATE	FIRST NAMED APPLICANT	ATTORNEY DOCKET NO.
06/[illegible]513	12/25/80	[illegible]	

JOHN P. WHITE
COOPER, DUNHAM, CLARK, GRIFFIN & MORAN
30 ROCKEFELLER PLAZA
NEW YORK, NY 10020

EXAMINER	
[illegible]PPLINGER, E	
ART UNIT	**PAPER NUMBER**
172	13

DATE MAILED: 8/13/82

This is a communication from the examiner in charge of your application.

COMMISSIONER OF PATENTS AND TRADEMARKS

☐ This application has been examined. ☑ Responsive to communication filed on 6/7/82 ☑ This action is made final

A shortened statutory period for response to this action is set to expire 3 month(s), ~~_____~~ days from the date of this letter.
Failure to respond within the period for response will cause the application to become abandoned. 35 U.S.C. 133

Part I THE FOLLOWING ATTACHMENT(S) ARE PART OF THIS ACTION:

1. ☐ Notice of References Cited by Examiner, PTO-892
2. ☐ Notice of Informal Patent Drawing, PTO-948
3. ☐ Notice of References Cited by Applicant, PTO-1449
4. ☐ Notice of Informal Patent Application, Form PTO-152
5. ☐ _____

Part II SUMMARY OF ACTION

1. ☑ Claims 1-9, 12-21, 23-27, 31-37, 39-54, 57-66, 71-73, 75-90, 93-114, 118, 124 and 125 are pending in the application.
   Of the above, claims 43, 44, 79, 80, 111, 112, 124 and 125 are withdrawn from consideration
2. ☐ Claims _____ have been cancelled.
3. ☐ Claims ~~45-54~~ 81-90 are allowed.
4. ☑ Claims 1-9, 12-21, 23-27, 31-37, ~~39-43~~, 45-54, 57-66, 71-73, ~~75-78~~, 93-114, 118 are rejected.
5. ☐ Claims _____ are objected to.
6. ☐ Claims _____ are subject to restriction or election requirement
7. ☐ The formal drawings filed on _____ are acceptable.
8. ☐ The drawing correction request filed on _____ has been ☐ approved. ☐ disapproved.
9. ☐ Acknowledgment is made of the claim for priority under 35 U.S.C. 119. The certified copy has
   ☐ been received. ☐ not been received. ☐ been filed in parent application, serial no. _____,
   filed on _____.
10. ☐ Since this application appears to be in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under Ex parte Quayle, 1935 C.D. 11; 453 O.G. 213.
11. ☐ Other

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GENE-CEN 083196

PTOL-326 (rev. 7-79)

EXAMINER'S ACTION

The disclosure is objected to as failing to provide an adequate disclosure of the claimed invention as required by the first paragraph of 35 U.S.C. 112. This paragraph of the statute, requires that the specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor of carrying out his invention. The claims are non-enabling for reasons of record. Applicants urge that claims 35-37 and 71-73 do not require that the protein from DNA I be produced. The claims recite "foreign" protein and thymidine kinase cannot be considered foreign. Thus, there is no enablement in the specification.

Applicants point to Lai et al and Graf et al as evidence that as of applicants' filing date others could use applicants' process to produce protein. It does not appear that Graf et al produced other protein. Moreover, although the references state that they used the transformation method of Wigler and Lai et al used cells from Wigler, the references may have require various manipulations to produce protein. Applicants cannot rely on another's ability to produce protein as evidence that anyone could have.

Applicants cite In re Strahilevitz and urge that the Examiner must provide reasons why one would not expect protein production. Applicants admit on pages 44 and 45 of their specification that β-globin had not been detected and that might stem from the absence of a functional ribosomal binding site. There are numerous factors involved in providing a functioning system, such as choosing genetic material which contains all the elements for transcription and translation, choosing a compatible plasmid and restriction enzyme. It is the Examiner's position that an undue amount of experimentation would be required given applicants' disclosure to produce protein.

Claims 31-37, 39-42, 71-73, 75-78, and 103-110 are rejected under 35 U.S.C. 112, first paragraph, for the reasons set forth in the preceding paragraph(s).

Claims 1-9, 12-21, 23-27, 31-37, 39-42, 45-54, 57-66, 71-73, 75-78, 81-90, 93-110, 113, 114 and 118 are rejected under 35 U.S.C. 103 as being unpatentable over Lai et al for reasons of record. Although, the invention is not identically disclosed or described as set forth in section 102 of Title 35 U.S.C., the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains.

Applicants' declarations under 37 CFR 1.131 are noted but as indicated during the telephone interviews of

February 23 and 27, 1982, evidence of conception by applicants is required. Mere statements by applicants are insufficient. See MPEP 715.07.

Moreover, the evidence provided cannot be relied upon to show evidence of production of protein. Claims 35-37 and 71-73 recite "foreign" protein and thymidine kinase is not foreign to the cells producing it.

Applicants argument concerning conception of their invention prior to the publication of Lai is not persuasive since applicants have not provided any evidence of such conception.

Claims 25-27, 65, 66, 70, 81-90, 93-97 and 100-110 are rejected under 35 U.S.C. 103 as being unpatentable over Mantei et al for reasons of record. Although, the invention is not identically disclosed or described as set forth in section 102 of Title 35 U.S.C., the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. The declarations under 37 CFR 1.131 are insufficient because applicants have not provided evidence of conception by applicants.

Claims 1, 2, 9, 12, 14, 16-21, 23, 25-27, 45, 46, 53, 54, 58, 60-66, 113, 114 and 118 are rejected under 35 U.S.C. 102(a) as clearly anticipated by Wigler et al W, V or

X for reasons of record because the invention was known or used by others in this country, or patented or described in a printed publication in this or a foreign country before the invention thereof by the applicant for patent. The declarations under 37 CFR 1.131 are insufficient because applicant have not provided evidence of conception by applicants.

Claims 3-8, 13, 15, 24, 31-37, 39-42, 47-52, 57, 59, 113, 114 and 118 are rejected under 35 U.S.C. 103 as being unpatentable over Wigler W, V or X for reasons of record. Although, the invention is not identically disclosed or described as set forth in section 102 of Title 35 U.S.C., the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. The declarations under 37 CFR 1.131 are insufficient. See above.

Claims 1, 2, 9, 12, 14, 16-18, 23, 25-27, 45, 46, 53, 54, 58, 60, 61, 64-66, 113, 114 and 118 are rejected under 35 U.S.C. 102(a) as clearly anticipated by Wold et al because the invention was known or used by others in this country, or patented or described in a printed publication in this or a foreign country before the invention thereof by the applicant for patent. The declarations under 37 CFR 1.131 are insufficient. See above.

Applicants urge that Wold et al teach transformation, not cotransformation. In the Results section, however, Wold et al clearly talk about cotransformation and transform with tk gene and additional DNA containing β-globin gene.

Claims 3-8, 13, 15, 19-21, 24, 30-37, 39-42, 47-52, 57, 59, 62, 63, 71-73 and 75-78 are rejected under 35 U.S.C. 103 as being unpatentable over Wold et al for reasons of record. Although, the invention is not identically disclosed or described as set forth in section 102 of Title 35 U.S.C., the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains.

Applicants arguments are answered in the preceding paragraph.

THIS ACTION IS MADE FINAL.

Kepplinger:sab
(703)557-3685
08-28-82

Esther M. Kepplinger
ESTHER M. KEPPLINGER
PRIMARY EXAMINER

UNITED STATES DEPARTMENT OF COMMERCE
Patent and Trademark Office
Address: COMMISSIONER OF PATENTS AND TRADEMARKS
Washington, D.C. 20231

SERIAL NUMBER	FILING DATE	FIRST NAMED APPLICANT	ATTORNEY DOCKET NO.
124513	2/25/80	Axel et al	16668A

EXAMINER	
E.M. Kepplinger	
ART UNIT	PAPER NUMBER
172	14

DATE MAILED:

EXAMINER INTERVIEW SUMMARY RECORD

All participants (applicant, applicant's representative, PTO personnel):

(1) John White (3) _____

(2) _____ (4) _____

Date of interview 10/6/82

Type: ☑ Telephonic ☑ Personal (copy is given to ☐ applicant ☐ applicant's representative).

Exhibit shown or demonstration conducted: ☑ Yes ☐ No. If yes, brief description: copy of In re Katz

Agreement ☐ was reached with respect to some or all of the claims in question. ☑ was not reached.

Claims discussed: all

Identification of prior art discussed: _____

Description of the general nature of what was agreed to if an agreement was reached, or any other comments: Mr. White urged that the tk gene inserted and produced is foreign to the transformed cells. He will present this argument in his response. It is probably sufficient. Mr. White will cancel claims to producing protein from DNA I + refile. Mr. White provided In re Katz which appears to be on point and state that an affidavit by the inventors that they are the sole inventors and that the other authors worked under their direction is sufficient evidence to eliminate those references.

(A fuller description, if necessary, and a copy of the amendments if available, which the examiner agreed would render the claims allowable must be attached. Also, where no copy of the amendments which would render the claims allowable is available, a summary thereof must be attached.)

Unless the paragraphs below have been checked to indicate to the contrary, A FORMAL WRITTEN RESPONSE TO THE LAST OFFICE ACTION IS NOT WAIVED AND MUST INCLUDE THE SUBSTANCE OF THE INTERVIEW (e.g., items 1–7 on the reverse side of this form). If a response to the last Office action has already been filed, then applicant is given one month from this interview date to provide a statement of the substance of the interview.

☐ It is not necessary for applicant to provide a separate reocrd of the substance of the interview.

☐ Since the examiner's interview summary above (including any attachments) reflects a complete response to each of the objections, rejections and requirements that may be present in the last Office action, and since the claims are now allowable, this completed form is considered to fulfill the response requirements of the last Office action.

Esther M. Kepplinger
Examiner's Signature

81-3679 PTOL-413 (rev. 1-81)

GENE-CEN 083202

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JAN 17 1983 PAT. & TRADEMARK OFF.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Richard Axel, et al.

Serial No.: 124,513 Examiner: Esther M. Kepplinger

Filed : February 25, 1980 Group Art Unit: 172

For : PROCESSES FOR INSERTING DNA INTO EUCARYOTIC CELLS AND FOR PRODUCING PROTEINACEOUS MATERIALS

DO NOT ENTER
OK TO ENTER
OK AS ENTERED

30 Rockefeller Plaza
New York, New York 10112

12 January 1983

Honorable Commissioner of Patents
and Trademarks
Washington, D. C. 20231

SIR:

OK to enter
Gwk 2/4/83

AMENDMENT AND

REQUEST FOR ONE MONTH EXTENSION

This Amendment is submitted in response to the outstanding Office Action issued in connection with the above-identified application on September 13, 1982. A response to that Office Action was due December 13, 1982. Applicants hereby request that the period for filing a response be extended one month to January 13, 1983, the date upon which this response is being filed. A check in the amount of $50. is enclosed as payment of the statutory fee for a one month extension of the time for filing a response.

Please amend the subject application as follows:

IN THE CLAIMS

Cancel claims 31-34; 39-42; 75-78 and 103-110.

01/26/83 124513 3 115 50.00 CK

REMARKS

In the September 13, 1982 Office Action the Examiner finally rejected claims 1-9; 12-21; 23-27; 31-37; 39-42; 45-54;

57-66; 71-73; 75-78; 81-90; 93-110; 114 and 118. In order to advance prosecution of the subject application applicants have hereinabove cancelled certain additional claims without prejudice to their right to reassert such claims at a later date. As a result of this Amendment the claims now pending in the subject application are the following: 1-9; 12-21; 23-27; 35-37; 45-54; 57-66; 71-73; 81-90; 93-102; 114 and 118.

Initially, applicants' undersigned attorney wishes to thank the Examiner for the courtesy of the interview held on October 6, 1982. Applicants' attorney thinks this interview was most helpful in clarifying the issues in the subject application.

In order to advance prosecution of this application applicants have cancelled claims 31-34; 39-42; 75-78 and 103-110 without prejudice to their right to present these claims in a subsequent application. By cancelling these claims applicants have eliminated all claims directed to the production of protein except claims 35-37 and 71-73. Claims 35-37 and 71-73 are broadly directed to the production of foreign protein by eucaryotic cells cotransformed by the processes to which the other pending claims are directed. As discussed during the interview, applicants understand that the Examiner's rejection of these claims was based upon her understanding that thymidine kinase is not foreign to the cotransformed cells. As pointed out during the interview, the thymidine kinase produced is foreign for two reasons. First, the thymidine kinase is produced from a gene obtained by herpes simplex virus (HSV) not from a gene obtained from the chromosonal DNA of a eucaryotic cell. Second, the cell being cotransformed is a tk^- cell, that is, a cell deficient in thymidine kinase. The first point finds support in the specification at numerous locations, e.g. page 13, lines 5-6 and page 22, lines 26-27. Likewise, the second point finds support in the specification at, inter alia,

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page 18, lines 2-3; page 22, lines 27-28 and page 32, lines 20-21.

During the interview the Examiner suggested applicants' argument concerning the fact that the tk gene and the thymidine kinase produced from it are foreign was probably sufficient to overcome the rejection. However, the Examiner did question whether there was an example in the application showing recovery of the thymidine kinase. In this regard, applicants direct the Examiner's attention to page 65, lines 27-34. As set forth there, cell extracts from cotransformed tk^+ cells were subjected to electrophoresis whereby the herpes simplex virus (HSV) thymidine kinase (tk) enzyme was separated from other components of the extract and characterized by its migration with an Rf value of 0.45. Thus, the foreign thymidine kinase was recovered.

From the interview, applicants understand that the Examiner's objection to the specification as non-enabling was based upon the lack of actual working examples of foreign protein production by cotransformed cells. As the preceding comments and the discussion during the interview indicated, this objection should not pertain to the claims remaining in the application since the thymidine kinase is foreign and since its production clearly is exemplified in the specification. In so far as the objection is directed to the production of proteins from DNA I, the ground for objection should be rendered moot by applicants' cancellation of such claims.

In view of the foregoing, applicants request that the Examiner reconsider and withdraw the grounds for rejection of claims 35-37 and 71-73 and for objection to the specification.

The second principal issue discussed during the inter view was the Examiner's continued rejection of claims as allegedly unpatentable over publications coauthored by appicants, namely, references W, V, X and Y. The Examiner reiterated her position

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that applicants must provide evidence that they alone conceived the invention and not the coauthors on the publications who are not named as coinventors on the subject application.

In response to the Examiner's position, applicants' undersigned attorney provided to the Examiner an unpublished copy of a recent decision of the Court of Customs and Patent Appeals, In re Katz. At this time applicants submit as Exhibit A hereto a copy of the published decision in In re Katz, 215 U.S.P.Q. 14 (1982) on which the most pertinent portions of the decision have been highlighted.

The decision in In re Katz is clearly on point concerning the extent to which a coauthor, patent applicant is required to show that a coauthor, non-applicant is not a coinventor. In the Katz decision the Court of Customs and Patent Appeals held that coinventorship cannot be presumed from coauthorship; that a requirement for disclaimers from coauthors who are not coinventors was erroneous; that an applicant's further oath or declaration stating that he is sole inventor of subject matter disclosed in a coauthored article and disclosed and claimed in a patent application and stating that other coauthors on the publication worked under his direction and supervision is sufficient to terminate inquiry by the Patent Office into inventorship; and finally that joint inventorship cannot be inferred in the presence of sworn statements to the contrary.

In the subject application applicants submitted declaration on November 9, 1981 and on June 7, 1982. In these declarations applicants declared, inter alia, the following:

1.	They solely conceived the invention disclosed and claimed in the application.	11/9: ¶2&3 6/7 : ¶4

2.	They are coauthors of the articles cited as references.	11/9 : ¶11 6/7 : ¶3
3.	They solely or through persons acting upon their supervision and direction reduced the invention to practice.	11/9 :¶4 6/7 : ¶6-10
4.	The invention disclosed and claimed in the application is the same as that disclosed in the articles.	11/9 : ¶5-9

Thus, applicants have provided a reasonable explanation for the fact that certain persons are named as coauthors but not coinventors and have reaverred that they alone made the invention disclosed and claimed in the application. Accordingly, applicants request that the Examiner reconsider and withdraw the various rejections based upon coauthored references W, V, X and Y.

The remaining rejections were based upon the teachings of Lai et al. and Mantei et al. From the interview applicants understand that these rejections will be reconsidered in view of applicants' previous declarations pursuant to 37 C.F.R. § 1.131, particularly in view of the following:

1. Claims directed to production of protein from DNA I have been cancelled at this time.
2. Applicants actually reduced to practice claimed embodiments of their invention prior to the effective date of the Mantei et al. article (Sept. 6, 1979) or the Lai et al. article (January 1980).
3. Mantei et al. concerned cotransformation with linked DNA I and DNA II, not unlinked DNAs.

30P

Accordingly, applicants request that the Examiner reconsider and withdraw the rejections based upon the alleged teachings of Mantei et al. or Lai et al.

In summary, applicants in a sincere effort to place this application in condition for allowance have cancelled claims directed to production of protein from DNA I. The remaining claims pending in the subject application are the following: 1-9; 12-21; 23-17; 35-37; 45-54; 57-66; 71-73; 81-90; 93-102; 114 and 118. Applicants maintain that these claims are now in condition for allowance and earnestly solicit their allowance. The major outstanding issues are believed to have been resolved as follows:

1. Thymidine kinase is foreign to the cotransformed cells producing it from foreign DNA.
2. Coauthors are not presumed to be coinventors and sworn statements by applicants providing a reasonable explanation for coauthors not being coinventors is a sufficient basis for removing the coauthored articles as references.
3. Separate evidence of conception is not required to remove as references the publications which applicants coauthored.
4. Separate evidence of conception is not required to remove as references the Mantei et al. or Lai et al. articles. Applicants' Rule 131 declarations are sufficient and establish actual reduction to practice prior to the effective dates of the references.

If a telephone interview would be of assistance in advancing prosecution of the application, applicants' undersigned attorney invites the Examiner to telephone him at the number provided.

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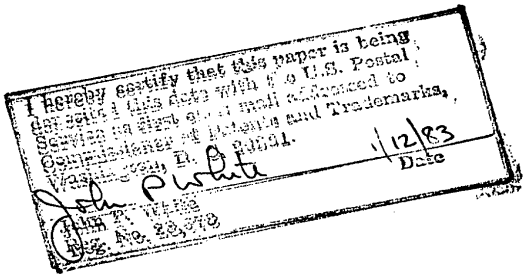
No fee, other than the $50 fee for a one month extension of time, is deemed necessary in connection with this Amendment. If a fee is required, authorization is hereby given to charge the amount of any such fee to Deposit Account No. 03-3125.

Respectfully submitted,

John P. White
Reg. No. 28678
Attorney for Applicants
212-977-9550



UNITED STATES DEPARTMENT OF COMMERCE
Patent and Trademark Office

Address: COMMISSIONER OF PATENTS AND TRADEMARKS
Washington, D.C. 20231

SERIAL NUMBER	FILING DATE	FIRST NAMED APPLICANT	ATTORNEY DOCKET NO.
08/124,513	09/20/93	HAEL	124513

JOHN P. WHITE
COOPER, DUNHAM, CLARK, GRIFFIN & MORAN
30 ROCKEFELLER PLAZA
NEW YORK, NY 10020

EXAMINER	
ART UNIT	PAPER NUMBER
	17

DATE MAILED: ent. 2-7-95

This is a communication from the examiner in charge of your application.

COMMISSIONER OF PATENTS AND TRADEMARKS

1. ☑ THIS IS AN ATTACHMENT TO THE NOTICE OF ALLOWANCE AND BASE ISSUE FEE DUE, PTOL 85.
2. ☑ All the claims being allowable, PROSECUTION ON THE MERITS IS CLOSED in this application. If not attached hereto, a Notice of Allowance or other appropriate communication will be sent in due course.

A. ☐ Note the attached PTO-152, Notice of Informality, which indicates that the declaration (or oath) is deficient and that a substitute is required. The substitute declaration (or oath) MUST BE SUBMITTED WITHIN THE THREE MONTH STATUTORY PERIOD SET FOR PAYMENT OF THE BASE ISSUE FEE IN THE "NOTICE OF ALLOWANCE AND BASE ISSUE FEE DUE" (PTOL-85), preferably with and attached to the base issue fee. Note that the statute does not permit extension of the three month period set for payment of the base issue fee. Failure to timely file the substitute declaration (or oath) will result in ABANDONMENT of the application. The transmittal letter accompanying the declaration (or oath) should indicate the following in the upper right hand corner:
Issue Batch Number; Date of the Notice of Allowance, and Serial Number.

B. ☐ Formal drawings are now required and MUST BE SUBMITTED WITHIN THE THREE MONTH STATUTORY PERIOD SET FOR PAYMENT OF THE BASE ISSUE FEE IN THE "NOTICE OF ALLOWANCE AND BASE ISSUE FEE DUE" (PTOL-85). Note that the statute does not permit extension of the three month period set to pay the base issue fee. Failure to timely submit the drawings will result in ABANDONMENT of the application. The drawings should be submitted as a separate paper with a transmittal letter which is addressed to the Official Draftsman and which indicates the following in the upper right hand corner:
Issue Batch Number; Date of the Notice of Allowance, and Serial Number.

C. ☑ The claims are allowed in view of:
   a. ☑ Applicant's communication filed 1/17.
   b. ☑ The interview summarized on the attached EXAMINER INTERVIEW SUMMARY RECORD, PTOL-413.
   c. ☑ The attached Examiner's Amendment.
   d. ☐ An Examiner's Amendment which will follow in due course.

D. ☑ The allowed claims are 1-9, 12-21, 23-27, 35-37, 45-54, 57-66, 71-73, 81-90, 93-102, 113, 114, 118.

3. ☐ Note the attached Examiner's Statement of Reasons for Allowance.

4. ☐ Note attached NOTICE OF REFERENCES CITED, PTO-892, which is part of this communication. The listed references are considered to be pertinent to the claimed invention, but the claims are deemed to be patentable thereover.

5. ☐ Note attached LIST OF ART CITED BY APPLICANT, PTO-1449.

6. ☐ The drawings filed on _____ ☐ are acceptable as filed. ☐ are acceptable subject to correction as indicated on the attached Notice re Drawings, PTO-948. In order to avoid ABANDONMENT of this application, correction is required. Corrections can only be made in accordance with the instructions set forth in the attached letter "INFORMATION ON HOW TO EFFECT DRAWING CHANGES", PTO-1474.

7. ☐ The ☐ proposed drawing correction and/or the ☐ proposed additional or substitute sheet(s) of drawings filed on _____ has (have) been approved by the examiner. Applicant is reminded that in order to avoid abandonment of this applicant, execution of the proposed changes or submission of additional or substitute drawings MUST be made in accordance with the instructions set forth in the letter, "INFORMATION ON HOW TO EFFECT DRAWING CHANGES", PTO-1474, attached to Paper No. _____.

8. ☐ The proposed drawing correction, filed _____, has been approved. However, the Patent and Trademark Office no longer makes drawing changes. It is now applicant's responsibility to ensure that the drawings are corrected. Corrections are required and MUST be effected in accordance with the instructions set forth on the attached letter "INFORMATION ON HOW TO EFFECT DRAWING CHANGES", PTO-1474.

9. ☐ In order to avoid ABANDONMENT, the drawing informalities noted on the Notice re Drawing, PTO-948, attached to Paper No. ____ must now be corrected. Applicant is reminded that the corrections can only be made in accordance with the instructions set forth in the letter "INFORMATION ON HOW TO EFFECT DRAWING CHANGES", PTO-1474, attached to the PTO-948.

10. ☐ Acknowledgment is made of the claim for priority under 35 U.S.C. 119. The certified copy has: ☐ been received. ☐ not been received.
☐ been filed in parent application, Serial No. _____ filed on _____.

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GENE-CEN 083210

PTOL - 37 (Rev. 8 - 82) NOTICE OF ALLOWABILITY

On page 37, line 3, corrected the spelling of --nitrocellulose--.

On page 39, line 31, corrected --nuclease--.

On page 52, line 28, corrected --expression--.

On page 64, line 13, corrected --demonstrated--.

Cancelled claims 43, 44, 79, 80, 112, 124 and 125 as non-elected claims.

In claim 118, line 1, corrected --foreign--.

The above changes were authorized by applicants' attorney, John White, in a telephone interview on February 4, 1983.

EMKEPPLINGER:hm
703-557-3685
2/7/83

Esther M. Keppinger
ESTHER M. KEPPLINGER
PRIMARY EXAMINER

230

UNITED STATES DEPARTMENT OF COMMERCE
Patent and Trademark Office
Address: COMMISSIONER OF PATENTS AND TRADEMARKS
Washington, D.C. 20231

SERIAL NUMBER	FILING DATE	FIRST NAMED APPLICANT	ATTORNEY DOCKET NO.
124513	2/25/80	Ayel et al	17668-A

EXAMINER	
E.M. Kepplinger	
ART UNIT	PAPER NUMBER
172	16

DATE MAILED:

EXAMINER INTERVIEW SUMMARY RECORD

All participants (applicant, applicant's representative, PTO personnel):

(1) Mr. John White (3) _____

(2) _____ (4) _____

Date of interview 2/4/83

Type: ☑ Telephonic ☐ Personal (copy is given to ☐ applicant ☑ applicant's representative).

Exhibit shown or demonstration conducted: ☐ Yes ☑ No. If yes, brief description: _____

Agreement ☑ was reached with respect to some or all of the claims in question. ☐ was not reached.

Claims discussed: 43, 44, 79, 80, 111, 112, 124, 125 and 118

Identification of prior art discussed: _____

Description of the general nature of what was agreed to if an agreement was reached, or any other comments: We agreed to cancel claims which were non-elected and also to spelling corrections. See Examiner's Amendment.

(A fuller description, if necessary, and a copy of the amendments, if available, which the examiner agreed would render the claims allowable must be attached. Also, where no copy of the amendments which would render the claims allowable is available, a summary thereof must be attached.)

Unless the paragraphs below have been checked to indicate to the contrary, A FORMAL WRITTEN RESPONSE TO THE LAST OFFICE ACTION IS NOT WAIVED AND MUST INCLUDE THE SUBSTANCE OF THE INTERVIEW (e.g., items 1–7 on the reverse side of this form). If a response to the last Office action has already been filed, then applicant is given one month from this interview date to provide a statement of the substance of the interview

☐ It is not necessary for applicant to provide a separate reocrd of the substance of the interview.

☐ Since the examiner's interview summary above (including any attachments) reflects a complete response to each of the objections, rejections and requirements that may be present in the last Office action, and since the claims are now allowable, this completed form is considered to fulfill the response requirements of the last Office action.

Esther M. Kepplinger GENE-GEN 083212
Examiner's Signature

81-3679 PTOL-413 (rev. 1-81)

85 (Rev. 8-82)

UNITED STATES DEPARTMENT OF COMMERCE
Patent and Trademark Office

Address : COMMISSIONER OF PATENTS AND TRADEMARKS
Washington, D.C. 20231

NOTICE OF ALLOWANCE AND ISSUE FEE DUE

JOHN P. WHITE
COOPER, DUNHAM, CLARK, GRIFFIN & MORAN
30 ROCKEFELLER PLAZA
NEW YORK, NY 10020

All communications regarding this application should give the serial number, date of filing, name of applicant, and batch number.

Please direct all communications to the Attention of "OFFICE OF PUBLICATIONS" unless advised to the contrary.

The application identified below has been examined and found allowable for issuance of Letters Patent. PROSECUTION ON THE MERITS IS CLOSED.

SC/SERIAL NO.	FILING DATE	TOTAL CLAIMS	EXAMINER AND GROUP ART UNIT		DATE MAILED
06/124,513	02/25/80	073	KEPPLINGER, E	172	02/14/83

First named applicant		
AXEL,	RICHARD	

TITLE OF INVENTION: PROCESSES FOR INSERTING DNA INTO EUCARYOTIC CELLS AND FOR PRODUCING PROTEINACEOUS MATERIALS

ATTY'S DOCKET NO.	CLASS-SUBCLASS	BATCH NO.	APPLN. TYPE	SMALL ENTITY	FEE DUE	DATE DUE
	435-006.000	083	UTILITY	NO	$500.00	05/16/83

The amount of the issue fee is specified by 37 C.F.R. 1.18 as follows: for an original or reissue patent, except for a design or plant patent, $500; for a design patent, $175; and for a plant patent, $250. If the applicant qualifies for and has filed a verified statement of small entity status in accordance with 37 C.F.R. 1.27, the issue fee is one-half the respective amount aforementioned. The issue fee due printed above reflects applicant's status as of the time of mailing this notice. A verified statement of small entity status may be filed prior to or with payment of the issue fee. However, in accordance with 37 C.F.R. 1.28, failure to establish status as a small entity prior to or with payment of the issue fee precludes payment of the issue fee in the amount so established for small entities and precludes a refund of any portion thereof paid prior to establishing status as a small entity.

THE ISSUE FEE MUST BE PAID WITHIN THREE MONTHS FROM THE MAILING DATE OF THIS NOTICE as indicated above. The application shall otherwise be regarded as ABANDONED. The issue fee will not be accepted from anyone other than the applicant; a registered attorney or agent; or the assignee or other party in interest as shown by the records of the Patent and Trademark Office. Where an authorization to charge the issue fee to a deposit account has been filed before the mailing of the notice of allowance, the issue fee is charged to the deposit account at the time of mailing of this notice in accordance with 37 C.F.R. 1.311. If the issue fee has been so charged, it is indicated above.

In order to minimize delays in the issuance of a patent based on this application, **this Notice may have been mailed prior to completion of final processing.** The nature and/or extent of the remaining revision or processing requirements may cause slight delays of the patent. In addition, **if prosecution is to be reopened, this Notice of Allowance will be vacated and the appropriate Office action will follow in due course.** If the issue fee has already been paid and prosecution is reopened, the applicant may request a refund or request that the fee be credited to a Deposit Account. However, applicant may wait until the application is either found allowable or held abandoned. If allowed, upon receipt of a new Notice of Allowance, applicant may request that the previously submitted issue fee be applied. If abandoned, applicant may request refund or credit to a Deposit Account.

In the case of each patent issuing without an assignment, the complete post office address of the inventor(s) will be printed in the patent heading and in the Official Gazette. If the inventor's address is now different from the address which appears in the application, please fill in the information in the spaces provided on PTOL-85b enclosed. If there are address changes for more than two inventors, enter the additional addresses on the reverse side of the PTOL-85b.

The appropriate spaces in the ASSIGNMENT DATA section of PTOL-85b must be completed in all cases. If it is desired to have the patent issue to an assignee, an assignment must have been previously submitted to the Patent and Trademark Office or must be submitted not later than the date of payment of the issue fee as required by 37 C.F.R. 1.334. Where there is an assignment, the assignee's name and address must be provided on the PTOL-85b to ensure its inclusion in the printed patent.

Advance orders for 10 or more printed copies of the prospective patent can be made by completing the information in Section 4 of PTOL-85b and submitting payment therewith. If use of a Deposit Account is being authorized for payment, PTOL-85c should also be forwarded. The order must be for at least 10 copies and must accompany the issue fee. The copies ordered will be sent only to the address specified in section 1 or 1A of PTOL-85b.

☒ Note attached communication from Examiner.

☐ This notice is issued in view of applicant's communication filed _____

IMPORTANT

ATTENTION IS DIRECTED TO 37 C.F.R. 1.334

THE PATENT WILL ISSUE TO APPLICANT UNLESS AN ASSIGNEE IS SHOWN IN

GENE-CEN 083213

$250.00-242

PTOL-85b (Rev. 8-82)

ISSUE FEE TRANSMITTAL

U.S. Department of Commerce
Patent and Trademark Office

This form is provided in lieu of a formal transmittal and should be used for transmitting the Issue Fee. Sections 1A through 4 must be completed as appropriate.

INVENTOR(S) ADDRESS CHANGE | SC/SERIAL NO.

INVENTOR'S NAME

Street Address

City, State and Zip Code

CO-INVENTOR'S NAME

Street Address

City, State and Zip Code

☐ Check if additional changes are on reverse side.

MAIL ROOM MAY 16 1983 PAT. & TRADE MARK OFF.

MAILING INSTRUCTIONS

All further correspondence including the Issue Fee Receipt the Patent, and advanced orders will be mailed to the addressee entered in section 1 on PTOL-85c, unless you direct otherwise by specifying the appropriate name and address in 1A below.

2A. The COMMISSIONER OF PATENTS AND TRADEMARKS is requested to apply the Issue Fee to the application identified below.

John P White

(Signature of party in interest of record) | (Date)

John P. White
Reg. No. 28,678

5/16/8

Note: The Issue Fee will not be accepted from anyone other than the applicant; a registered attorney or agent; or the assignee or other party in interest as shown by the records of the Patent and Trademark Office.

	SC/SERIAL NO.	FILING DATE	TOTAL CLAIMS	EXAMINER AND GROUP ART UNIT		DATE MAILED
	06/124,513	02/25/80	073	KEPPLINGER, E	172	02/14/83

First Named Applicant: AXEL, RICHARD

TITLE OF INVENTION: PROCESSES FOR INSERTING DNA INTO EUCARYOTIC CELLS AND FOR PRODUCING PROTEINACEOUS MATERIALS

	ATTY'S DOCKET NO.	CLASS-SUBCLASS	BATCH NO.	APPLN. TYPE	SMALL ENTITY	FEE DUE	DATE DUE
		435-006.000	083	UTILITY	~~NO~~ Yes*	~~$500.00~~ $250.00	05/16/83

1A. Further correspondence to be mailed to the following:

John P. White, Esq.
Cooper, Dunham, Clark, Griffin & Moran
30 Rockefeller Plaza
New York, New York 10112

S

2B. For printing on the patent front page, list the names of not more than 3 registered patent attorneys or agents OR, alternatively, the name of a firm having as a member a registered attorney or agent. If no name is listed, no name will be printed.

1 John P. White
2
3

DO NOT USE THIS SPACE

05/23/83 124513 2 242 250.00 CK

~~50400 05/23/83 124513~~ 03-3125 1 501 ~~33.00CH~~

3. ASSIGNMENT DATA (print or type)

A. (1) ☐ This application is NOT assigned.
(2) ☒ Assignment previously submitted to the Patent and Trademark Office.
(3) ☐ Assignment submitted herewith.

B. For Printing On The Patent: (Unless an assignee is identified below, no assignee data will appear on the patent. Inclusion of assignee data below is only appropriate when an assignment has been previously submitted to the PTO or is submitted herewith. Completion of this form is NOT a substitute for filing of an assignment as required by 37 C.F.R. 1.334).

(1) NAME OF ASSIGNEE: THE TRUSTEES OF COLUMBIA UNIVERSITY IN THE CITY OF NEW YORK

(2) ADDRESS: (City & State or Country)
New York, New York

(3) STATE OF INCORPORATION, IF ASSIGNEE IS A CORPORATION:
New York

*small entity form enclosed

4. The following fees are enclose:
☒ Issue fee ☐ Advanced order ☐ Assignment recording

The following fees should be charged to deposit acc. no. 03-3125
(PTOL-85c or additional copy of PTOL-85b must be enclosed)

☐ Issue fee
☒ Advanced order
☐ Assignment recording

Number of advanced order copies requested. 33
(must be for 10 or more copies)

GENE-CEN 083214

TRANSMIT THIS FORM WITH FEE

Applicant or Patentee: AXEL, ET AL.
Serial or Patent No.:
Filed or Issued: February 25, 1980
For: PROCESSES FOR INSERTING DNA INTO EUCARYOTIC CELLS AND FOR PRODUCING PROTEINACEOUS MATERIALS

Attorney's Docket No: 17658-

MAIL ROOM MAY 16 1983

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS
(37 CFR 1.9(f) and 1.27(d)) - NONPROFIT ORGANIZATION

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:

NAME OF ORGANIZATION THE TRUSTEES OF COLUMBIA UNIVERSITY IN THE CITY OF NEW YORK
ADDRESS OF ORGANIZATION Broadway & West 116th Street
New York, New York 10027

TYPE OF ORGANIZATION

[x] UNIVERSITY OR OTHER INSTITUTION OF HIGHER EDUCATION
[ ] TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE (26 USC 501(a) and 501(c)(3))
[ ] NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED STATES OF AMERICA
(NAME OF STATE)
(CITATION OF STATUTE)
[ ] WOULD QUALIFY AS TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE (26 USC 501(a) and 501(c)(3)) IF LOCATED IN THE UNITED STATES OF AMERICA
[ ] WOULD QUALIFY AS NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED STATES OF AMERICA IF LOCATED IN THE UNITED STATES OF AMERICA
(NAME OF STATE)
(CITATION OF STATUTE)

I hereby declare that the nonprofit organization identified above qualifies as a nonprofit organization as defined in 37 CFR 1.9(e) for purposes of paying reduced fees under section 41(a) and (b) of Title 35, United States Code with regard to the invention entitled PROCESSES FOR INSERTING DNA INTO EUCARYOTIC CELLS AND FOR PRODUCING PROTEINACEOUS MATERIALS by inventors, Richard Axel, Michael H. Wigler and Saul J. Silverstein, described in

[ ] the specification filed herewith
[x] application serial no. 124,513, filed February 25, 1980
[ ] patent no. , issued

I hereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization with regard to the above identified invention.

If the rights held by the nonprofit organization are not exclusive, each individual, concern or organization having rights to the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who could not qualify as a small business concern under 37 CFR 1.9(d) or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e). *NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

NAME N/A
ADDRESS
[ ] INDIVIDUAL [ ] SMALL BUSINESS CONCERN [ ] NONPROFIT ORGANIZATION

NAME
ADDRESS
[ ] INDIVIDUAL [ ] SMALL BUSINESS CONCERN [ ] NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

GENE-CEN-083215

UNITED STATES DEPARTMENT OF COMMERCE
Patent and Trademark Office

Address: COMMISSIONER OF PATENTS AND TRADEMARKS
Washington, D.C. 20231

SERIAL NUMBER	FILING DATE	FIRST NAMED APPLICANT	ATTORNEY DOCKET NO.
124,513	02-25-80	Richard Axel, et al	17668A

John P. White
Cooper, Dunham, Clark, Griffin & Moran
30 Rockefeller Plaza
New York, NY 10020

EXAMINER	
E. Kepplinger	
ART UNIT	PAPER NUMBER
174	18

MAILED
JUL 5 1983
DATE MAILED:

ext. 6130-8.
B.H.

This is a communication from the examiner in charge of your application.

COMMISSIONER OF PATENTS AND TRADEMARKS

GROUP 170

1. ☐ THIS IS AN ATTACHMENT TO THE NOTICE OF ALLOWANCE AND BASE ISSUE FEE DUE, PTOL 85.
2. ☐ All the claims being allowable, PROSECUTION ON THE MERITS IS (OR REMAINS) CLOSED in this application. ~~If not attached hereto, a Notice of Allowance or other appropriate communication will be sent in due course.~~

   A. ☐ Note the attached PTO-152, Notice of Informality, which indicates that the declaration (or oath) is deficient and that a substitute is required. The substitute declaration (or oath) MUST BE SUBMITTED WITHIN THE THREE MONTH STATUTORY PERIOD SET FOR PAYMENT OF THE BASE ISSUE FEE IN THE "NOTICE OF ALLOWANCE AND BASE ISSUE FEE DUE" (PTOL-85), preferably with and attached to the base issue fee. Note that the statute does not permit extension of the three month period set for payment of the base issue fee. Failure to timely file the substitute declaration (or oath) will result in ABANDONMENT of the application. The transmittal letter accompanying the declaration (or oath) should indicate the following in the upper right hand corner: Issue Batch Number; Date of the Notice of Allowance, and Serial Number.

   B. ☐ Formal drawings are now required and MUST BE SUBMITTED WITHIN THE THREE MONTH STATUTORY PERIOD SET FOR PAYMENT OF THE BASE ISSUE FEE IN THE "NOTICE OF ALLOWANCE AND BASE ISSUE FEE DUE" (PTOL-85). Note that the statute does not permit extension of the three month period set to pay the base issue fee. Failure to timely submit the drawings will result in ABANDONMENT of the application. The drawings should be submitted as a separate paper with a transmittal letter which is addressed to the Official Draftsman and which indicates the following in the upper right hand corner: Issue Batch Number; Date of the Notice of Allowance, and Serial Number.

   C. ☑ ~~The claims are allowed in view of~~ NOTE

      a. ☐ Applicant's communication filed _____.

      b. ☐ The interview summarized on the attached EXAMINER INTERVIEW SUMMARY RECORD, PTOL-413.

      c. ☑ The attached Examiner's Amendment.

      d. ☐ An Examiner's Amendment which will follow in due course.

   D. ☐ The allowed claims are _____

3. ☐ Note the attached Examiner's Statement of Reasons for Allowance.

4. ☐ Note attached NOTICE OF REFERENCES CITED, PTO-892, which is part of this communication. The listed references are considered to be pertinent to the claimed invention, but the claims are deemed to be patentable thereover.

5. ☐ Note attached LIST OF ART CITED BY APPLICANT, PTO-1449.

6. ☐ The drawings filed on _____ ☐ are acceptable as filed. ☐ are acceptable subject to correction as indicated on the attached Notice re Drawings, PTO-948. In order to avoid ABANDONMENT of this application, correction is required. Corrections can only be made in accordance with the instructions set forth in the attached letter "INFORMATION ON HOW TO EFFECT DRAWING CHANGES", PTO-1474.

7. ☐ The ☐ proposed drawing correction and/or the ☐ proposed additional or substitute sheet(s) of drawings filed on _____ has (have) been approved by the examiner. Applicant is reminded that in order to avoid abandonment of this applicant, execution of the proposed changes or submission of additional or substitute drawings MUST be made in accordance with the instructions set forth in the letter, "INFORMATION ON HOW TO EFFECT DRAWING CHANGES", PTO-1474, attached to Paper No. _____

8. ☐ The proposed drawing correction, filed _____, has been approved. However, the Patent and Trademark Office no longer makes drawing changes. It is now applicant's responsibility to ensure that the drawings are corrected. Corrections are required and MUST be effected in accordance with the instructions set forth on the attached letter "INFORMATION ON HOW TO EFFECT DRAWING CHANGES", PTO-1474.

9. ☐ In order to avoid ABANDONMENT, the drawing informalities noted on the Notice re Drawing, PTO-948, attached to Paper No. ____, must now be corrected. Applicant is reminded that the corrections can only be made in accordance with the instructions set forth in the letter "INFORMATION ON HOW TO EFFECT DRAWING CHANGES", PTO-1474, attached to the PTO-948.

10. ☐ Acknowledgment is made of the claim for priority under 35 U.S.C. 119. The certified copy has; ☐ been received. ☐ not been received.

    ☐ been filed in parent application, Serial No. _____ filed on _____.

232

GENE-CEN 083216

PTOL - 37 (Rev. 8 - 82)

NOTICE OF ALLOWABILITY

On page 1 of the abstract, cancelled lines 15-21.

On page 1 of the abstract, line 25, deleted "which includes" and inserted --including--.

In line 26, after "materials" deleted the period and the remainder of the line.

In line 27, delete "genes is accomplished".

On page 2 of the abstract, line 5, deleted "Eucaryotic Cells into which".

On page 2 of the abstract, deleted lines 6-11.

E.KEPPLINDER:vb

703-557-3032

5/30/83

Esther M. Kepplinger

ESTHER M. KEPPLINGER
PRIMARY EXAMINER

17668-A JPW

CRC #19 89

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants : Richard Axel, et al.

Serial No. : 124,513

Filed : February 25, 1980

For : PROCESSES FOR INSERTING DNA INTO EUCARYOTIC CELLS AND FOR PRODUCING PROTEINACEOUS MATERIALS

PATENT NO. : 4,399,216

DATED : August 16, 1983

Group Art Unit 175
Examiner: E. Kepplinger

30 Rockefeller Plaza
New York, New York 10112

March 20, 1984

APPROVED
AS INDICATED
FEB 11 1985
K.C. Mason
FOR THE COMMISSIONER OF
PATENTS & TRADEMARKS

Hon. Commissioner of Patents
and Trademarks
Washington, D.C. 20231

Sir:

REQUEST FOR CERTIFICATE OF CORRECTION UNDER 37 C.F.R. § 1.322

Attached please find one (2) page of Patent Office Form PTO-1050 indicating errors noted in proofreading the above-identified patent. These errors were introduced by the Patent Office in the course of printing the patent. The corrections are as indicated in the originally filed application.

The enclosed Certificate of Correction was not necessitated by error on Applicants' behalf. It is, therefore, requested that

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GENE-CEN 083218

a Certificate of Correction be issued in accordance with the provisions of 37 C.F.R. § 1.322.

No fee is deemed necessary in connection with issuance of this Certificate of Correction. If a fee is necessary, authorization is hereby given to charge Deposit Account No. 03-3125 with the amount of any such fee.

Respectfully submitted,

John P White

John P. White, Reg. No. 28,678
Attorney for Applicants

(212) 977-9550

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 4,399,216

DATED : August 16, 1983

INVENTOR(S) : Richard Axel, Michael H. Wigler, Saul J. Silverstein

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Col. 3, line 35, after "hormones" insert a comma --(,)--.

Col. 8, line 51, after "pBR" insert --322--.

Col. 10, line 26, after "Φ" insert --X--.

Col. 12, line 60, "transforments" should read --transformants--.

Col. 15, line 21, "ribbit" should read --rabbit--.

Col. 17, line 68, "therefor" should read --therefore--.

Col. 18, line 58, "exceding" should read --exceeding--.

Col. 22, line 20, "tk^{30}" should read --tk^{+}--.

Col. 23, line 57, "=P" should read --^{32}p--.

Col. 30, line 15, "μ/ml" should read --μg/ml--.

Col. 30, line 52, "μ/ml" should read --μg/ml--.

Col. 34, line 57, "Bgl/32" should read --Bgl--.

Col. 34, line 59, "P-labeled" should read --^{32}p-labeled--.

Col. 35, line 21, "anenue" should read --avenue--.

GENE-CEN 083220

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 4,399,216 Page 2 of 2

DATED : August 16, 1983

INVENTOR(S) : Richard Axel, Michael H. Wigler, Saul J. Silverstein

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Col. 37, line 57, "ffrom" should read --from--.

Col. 38, line 60, after "A29" insert --DNA--.

Col. 44, line 68, "does" should read --codes--.

Signed and Sealed this

Twenty-sixth Day of *March 1985*

[SEAL]

Attest:

DONALD J. QUIGG

Attesting Officer *Acting Commissioner of Patents and Trademarks*

[redacted]83221

U.S. DEPARTMENT OF COMMERCE
Patent and Trademark Office
Address: COMMISSIONER OF PATENTS AND TRADEMARKS
Washington, D.C. 20231

PATENT NO.	PATENT DATE
4,399,216	08-16-83

PATENTEE
Richard Axel, et al

John P. White
Cooper, Dunham et al
30 Rockefeller Plaza
New York, NY 10112

#20

MAILING DATE
JUN 11 1984

NOTIFICATION OF APPROVAL IN-PART OF CERTIFICATE OF CORRECTION

The Certificate of Correction requested in the patent identified above has been APPROVED with the exceptions indicated below. The remaining errors will be corrected as requested. The Certificate, so modified, will be issued on SEP 11 1984

A. [X] THE CHANGES BELOW CANNOT BE INCLUDED IN THE CERTIFICATE SINCE THE REQUEST WAS FILED UNDER RULE 322.

1. [X] Column 44, line 17, is printed in accordance with the record.

(a) [ ] The change referred to was initialed and dated by applicant before execution of the application papers.

2. [ ] In column _____, line _____, the error resulted from applicant's failure to comply with Rule 121(a), in that the precise point of entry of the amendment was omitted.

3. [ ] In column _____, line _____, the alleged error is due to applicant's failure to comply with Rule 121(b), wherein provision is made for use of brackets, instead of parentheses, to cancel subject matter and for the use of interlineations to indicate new subject matter.

4. [ ] Omission of the priority data from the patent resulted from applicant's failure to fully comply with 35 U.S.C. 119, in that:

(a) [ ] The priority data was omitted from the oath, or declaration.

(b) [ ] The claim for priority was not included in the application papers.

(c) [ ] The certified copy of the foreign application was not filed.

5. [ ] The assignment data is printed in the patent in accordance with PTO 76-13b, submitted by applicant at time of payment of the base issue fee, as follows:

6. [ ] In column _____, line _____, the error arose because Rule 52(b) was not complied with. Consequently, words on top of certain pages were obliterated where those pages were placed in the file jacket, causing the Office to provide what appeared to be the proper words.

B. [ ] THE REQUEST HAS BEEN CHANGED AS SHOWN BELOW TO COMPLY WITH THE RECORD:

1. [ ] Since it is not normally the practice of the Office to reprint figures of the drawings, the following narrative description of Fig(s). _____ as suggested by the examiner, will be included:

Form PTOL-404 (rev. 10/80) (over)

GENE-CEN 083222

NOTICE RE: CERTIFICATES OF CORRECTION Paper No. 19 1/2

DATE: 9-24-84

TO : Director, Group 170

SUBJECT: Certificate of Correction Request in Patent No. 4399216

A response to the following question(s) is requested with respect to the accompanying request for a certificate of correction.

Atty's Error

1. ☒ Would the change(s) requested under 37 CFR 1.323 constitute new matter or require reexamination of the application?

2. ☐ Applicant disagrees with change(s) initialed and dated by Examiner in lieu of an Examiner's Amendment. Should the change request be granted?

3. ☐ With respect to the change(s) requested should the patent read as shown in the certificate of correction?

4. ☐ If the amendment filed _____ had been considered by the Examiner, would the amendment have been entered?

PLEASE RESPOND WITHIN 7 DAYS AND RETURN THE FILE TO ROOM 2-10C26.

B-Jones
Patent Assistant

TO: CERTIFICATES OF CORRECTION BRANCH DATE: October 18, 1984

The decision regarding the change(s) requested in the certificate of correction is shown below.

1.	☒ Yes	☐ No	☒ Comments below
2.	☐ Yes	☐ No	☐ Comments below
3.	☐ Yes	☐ No	☐ Comments below
4.	☐ Yes	☐ No	☐ Comments below

☒ Comments Proposed change to Col. 44, line 17 would make claim 30 a duplicate of claim 20.

Acting Director _____ Group 170

GENE-CEN 083223

PTOL-306 (rev. 5/78) U.S. DEPARTMENT of COMMERCE-Patent and Trademark Office

UNITED STATES DEPARTMENT OF COMMEI
Patent and Trademark Office
Address : COMMISSIONER OF PATENTS AND TRADEMAR
Washington, D.C. 20231

FEB 11 1985

Patent No. 4,399,216
Richard Axel, et al.
Issued: August 16, 1983
Processes for inserting DNA into eucaryotic cells and, etc.

Re: Request for Certificate of Correction

Consideration has been given your request for the issuance of a certificate of correction in the above-identified patent under Rule 322.

A certificate of correction will be issued to correct the errors noted in your request, except for the alleged error in column 44, line 17.

Respecting the alleged error in column 44, line 17, your request was referred to the Group Director who reports as follows:

"Proposed change to Col. 44, line 17 would make claim 30 a duplicate of claim 20."

Ruth C. Mason
Ruth C. Mason
Manager, Publishing Division
Certificates of Correction Branch
(703) 557-0709

John P. White
Cooper, Dunham, et al.
30 Rockefeller Plaza
New York, New York 10112

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Correction Certificate

PAGE 1 of 2

MISSING

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 4,399,216 Page 2 of 2

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Twenty-sixth Day of *March 1985*

[SEAL]

Attest:

DONALD J. QUIGG

Attesting Officer *Acting Commissioner of Patents and Trademarks*

241

GENE[illegible]N 083226

(3/85)

SOLICITOR

Commissioner of Patents and Trademarks
Washington, D.C. 20231

NOV 29 1990
U.S. PATENT & TRADEMARK OFFICE

REPORT ON THE FILING OR DETERMINATION OF AN ACTION REGARDING A PATENT

In compliance with the Act of July 19, 1952 (66 Stat. 814; 35 U.S.C. 290) you are hereby advised that a court action has been filed on the following patent(s) in the U.S. District Court:

DOCKET NO.	DATE FILED	U.S. DISTRICT COURT
90-677	11/20/90	DELAWARE

PLAINTIFF	DEFENDANT
THE TRUSTEES OF COLUMBIA UNIVERSITY IN THE CITY OF NEW YORK	GENETICS INSTITUTE, INC

	PATENT NO.	DATE OF PATENT	PATENTEE
1	4,399,216	8/16/83	The Trustees of Columbia University
2	4,634,665	1/6/87	The Trustees of Columbia University in the City of New York
3			
4			
5			

In the above-entitled case, the following patent(s) have been included:

INCLUDED BY ☐ Amendment ☐ Answer ☐ Cross Bill ☐ Other Pleading

	DATE OF PATENT	PATENTEE
3		
4		
5		

In the above-entitled case, the following decision has been rendered or judgment issued:

DECISION/JUDGMENT

CLERK	(BY) DEPUTY CLERK	DATE
John R. McAllister, Jr.,	Brian K Blackwell	11/26/90

Copy 1 — Upon initiation of action, mail this copy to Commissioner

GENE-CEN 083227